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EFFECT OF HORMONES ON THE TRICHLOROACETIC ACID EXTRACTABLE GLYCOGEN IN SKELETAL MUSCLES[1]

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COLD 10% trichloroacetic acid (TCA) removes from homogenized liver and muscle tissue respectively, a relatively constant amount of glycogen, somewhat less than the total amount present as determined by the usual hot alkali method of extraction (Bloom *et al.*, 1951). Whenever glycogenolytic agents (epinephrine, nerve stimulation) reduce the glycogen in muscle tissue, it is the TCA extractable fraction which decreases markedly (Bloom and Knowlton, 1953). These investigators attribute a "labile" quality to the TCA soluble fraction, in contrast to the more resistant "residual" glycogen fraction which is relatively unaffected after mild stimulation of these tissues by glycogenolytic agents.

Since both cortisone and growth hormone vary in their ability to influence glycogen deposition in different muscles (Leonard, 1953) and in their ability to inhibit the glycogenolytic action of epinephrine (Wortman and Leonard, 1953; Leonard and Ringler, 1954), a possible explanation of these phenomena was sought in a study of the changes in the "labile" and "residual" glycogen fractions in muscles of rats treated with these hormones. Theoretically, the hormone which increases the "residual" glycogen should be the one that also would inhibit epinephrine glycogenolysis. The following experiments were designed to test this hypothesis.

METHODS

Normal and hypophysectomized adult female rats (210–240 gm.) were employed. A period of at least 8 days elapsed after hypophysectomy before any subsequent treatment was begun and all animals were starved 24 hours before removing the muscles.

Epinephrine was injected in doses of 5 μg. and 20 μg. per 100 gm. body weight, intra-

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peritoneally, 1 hour before autopsy. Cortisone acetate[2] (2 mg. per day for 3 days) was given subcutaneously; growth hormone[2] (2 mg. per day for 3 days) was given in divided doses twice daily, intraperitoneally.

The procedure for extracting the glycogen was the same as described by Bloom *et al.* (1951) except for minor differences. One *rectus femoris* muscle and the whole diaphragm were removed from rats under nembutal anesthesia and quickly frozen between two pieces of carbon dioxide ice. While frozen, each muscle was wrapped in aluminum foil and kept frozen in a carbon dioxide ice-acetone mixture until ready for extraction. Each muscle was divided, one half was weighed on a torsion balance and digested in 2 cc. of hot 30% KOH for 30 minutes. The other frozen half was weighed, transferred back to a block of dry ice, cut into small fragments with a razor blade and the fragments transferred to an ice cold Elvehjem-Potter homogenizer containing 2–3 cc. of cold 10% trichloroacetic acid. The homogenizer was kept at all times in a salt-ice bath and after grinding, the homogenate was transferred to a cold centrifuge tube. The homogenizer was rinsed with cold TCA and the washings combined. In all, about 3 cc. of TCA per 100 mg. of tissue was used in the extraction. The homogenate was centrifuged in iced centrifuge cups for 10 minutes and the clear extract transferred to a graduated centrifuge tube. A 1.1 volume of cold 95% alcohol was added to the extract, mixed, and the mixture allowed to stand overnight in the refrigerator. The precipitated glycogen was centrifuged and after discarding the supernatant fluid, the tubes were allowed to drain thoroughly. The glycogen was dissolved in a small volume of water and and an aliquot taken for glycogen determination by the anthrone method of Seifter *et al.* (1950).

The muscle tissue digested in KOH was treated as previously described (Leonard, 1953) and the *total glycogen* determined by the anthrone method. Glycogen is reported as mg. of glucose per 100 gm. of muscle, wet weight. The "residual" glycogen was calculated as the difference between the total KOH extractable and the TCA extractable glycogen. The TCA fraction is also presented in the table as a percent of the total glycogen.

RESULTS

The results presented under part A, Table 1, were obtained during the hot summer months, June-August, and under B, during the late fall, and the data will be considered separately in making comparisons within each group. In normal female rats, it was observed that the total glycogen and the TCA soluble glycogen in the rectus femoris muscle, was greater than that found in the diaphragm but the residual glycogen was distinctly higher in the diaphragm. Furthermore, the percent of TCA soluble glycogen was always higher in the leg muscle than in the diaphragm in this and in all subsequent experiments in which the total glycogen content of the two muscles was made to vary by hormonal changes in the animal.

The injection of 5μg. doses of epinephrine into normal rats failed to lower the total glycogen or the fractions thereof in this leg muscle but it did in the diaphragm (Table 1-A). According to previous experience, this amount of epinephrine should have been adequate to effect a change in this leg muscle (Leonard and Ringler, 1954). It is quite possible that the stress of

[2] Cortisone acetate was donated by the Schering Corporation, Bloomfield, New Jersey and the growth hormone preparation (Rx099862) by Parke, Davis and Company, Detroit, Michigan.

heat on the rats produced sufficient adrenal cortical stimulation to inhibit the glycogenolytic action of epinephrine on the leg muscles, but since this inhibitory action of the cortical hormones does not occur in the diaphragm (Wortman and Leonard, 1953), the usual glycogenolytic effects were observed in this muscle.

When epinephrine was given in 20 μg. doses to normal rats under more favorable conditions, a decrease in the total, the TCA soluble fraction and percent of TCA soluble glycogen in the leg muscle occurred, but the residual glycogen remained unchanged (Table 1-B). This confirms the results of Bloom *et al.* (1951). However, in the diaphragms of the same rats, not only was the total and the percent of acid soluble glycogen reduced but unlike the situation in the leg muscles, the "residual" fraction was also decreased.

The injection of growth hormone into normal rats raised the total glycogen and the TCA soluble fraction significantly but the increase in the residual glycogen was not statistically significant (Table 1-B). The glycogen in the diaphragm was unchanged. Following cortisone treatment, a marked rise of the residual and acid soluble glycogen in the leg muscle occurred together with the expected increase in the total amount, but the percent of TCA soluble glycogen remained unchanged. In the diaphragm, the "residual" fraction did not increase above the control level but the percent of acid soluble glycogen did (Table 1-B).

A further study was made using hypophysectomized rats. As previously reported, (Russell, 1936), a decrease in the total glycogen occurred in the muscles of the starved hypophysectomized rats when compared to the levels found in the intact controls (Table 1-A). In the leg muscle, the loss was in the acid soluble fraction. In the diaphragm, the loss was in both fractions.

TABLE 1. GLYCOGEN FRACTIONS EXTRACTED FROM SKELETAL MUSCLE UNDER DIFFERENT HORMONAL CONDITIONS

Experiment	No. of rats	Rectus femoris*				Diaphragm*			
		Glycogen, mg. %			% TCA sol. glycogen	Glycogen, mg. %			% TCA sol. glycogen
		Total	TCA sol.	Residual		Total	TCA sol.	Residual	
				A					
Normal control	21	358±14	301±13	58± 9	84±2.2	249± 8	111± 6	138±6	44±2.0
Normal +Epineph. 5 μg/100 gm.	17	326±12	272±18	54± 6	83±2.3	137± 9	51± 6	85±6	37±3.0
Hypsect. control	27	202± 8	141± 6	61± 5	70±2.3	190± 8	77± 6	113±4	40±1.9
Hypsect. +Epineph. 5 μg/100 gm.	22	172± 6	102± 5	70± 4	59±2.1	131± 7	40± 5	91±4	30±2.2
Hypsect. +Cortisone	9	431±33	338±31	93± 9	78±2.1	303±16	163±15	140±3	53±2.0
Hypsect. +Growth h.	7	382±13	266±14	117±12	70±3.0	263± 9	140± 8	123±4	53±1.8
				B]					
Normal control	7	380±19	295±17	85±10	78±2.1	291±19	140±13	151±7	48±1.6
Normal +Epineph. 20 μg/100 gm.	10	185± 6	103± 9	83± 6	55±3.6	84± 6	17±18	67±6	21±3.0
Normal +Growth h.	7	475±20	371±17	104± 9	78±1.4	283±15	146±10	137±6	52±1.3
Normal +Cortisone	7	572±34	435±22	137±17	77±1.9	347±16	197±11	149±7	57±1.2

* Results are given with the standard error of the mean.

When glycogenolysis was induced in the hypophysectomized rats by 5 μg. doses of epinephrine, a decrease in the total and acid soluble fractions occurred in the leg muscles, when compared to the levels in the hypophysectomized controls, but the "residual" glycogen remained unchanged. In the diaphragm, however, the loss of glycogen was observed in both fractions.

Cortisone treatment of hypophysectomized rats increased both glycogen fractions and the percent of acid soluble glycogen by a statistically significant amount in both muscles. Growth hormone injections increased both glycogen fractions most markedly in the leg muscle without altering the percent of the acid soluble fraction. In the diaphragm, only the TCA soluble glycogen fraction and the percent of the acid soluble fraction was increased significantly.

DISCUSSION

The above experiments were made in an attempt to find an explanation for previous observations that cortisone and growth hormone inhibit the glycogenolytic action of epinephrine only on certain skeletal muscles of the rat (Wortman and Leonard, 1953), (Leonard and Ringler, 1954). The experiments of Bloom *et al.* (1951, 1953) on liver and the gastrocnemius muscle, and Adrouny and Russell (1954) on heart muscle, indicate that the TCA soluble glycogen fraction is most sensitive to change when alterations in the total glycogen level in the tissues are induced. The residual glycogen on the other hand, is resistant to change, particularly to the action of epinephrine. This suggested the possibility that cortisone and growth hormone might convert more of the glycogen into the "residual" form, resistant to glycogenolysis.

To some extent, the observations agreed in principle with the hypothetical expectations. Cortisone, which inhibits epinephrine glycogenolysis in the rectus femoris muscle but not in the diaphragm of normal rats (Wortman and Leonard, 1953) increased the residual glycogen in this leg muscle but not in the diaphragm. Growth hormone failed to inhibit epinephrine glycogenolysis in this leg muscle of normal rats (Leonard and Ringler, 1954) and neither did it increase the residual glycogen significantly. In hypophysectomized rats, both cortisone and growth hormone, at optimum dose levels, inhibited epinephrine glycogenolysis in the rectus femoris muscle (Leonard and Ringler, 1954) and in both instances, the residual glycogen was increased by significant amount.

In spite of this, there are two reasons why these observations cannot explain the results of the previous experiments. (1) The increase in the residual glycogen was not sufficiently large to account for the inhibition obtained. (2) In the diaphragm of hypophysectomized rats, neither cortisone nor growth hormone inhibited epinephrine glycogenolysis yet both hormones, but particularly cortisone, increased the residual glycogen to some extent.

There is no doubt that when changes in the total glycogen levels are produced in certain skeletal muscles by hormonal treatment, it is usually the TCA soluble fraction which is more labile, thus confirming the conclusions of Bloom *et al.* (1951). Adrouny and Russell (1954) indicate this is also true for heart muscle but Merrick and Meyer (1954) succeeded in increasing the residual glycogen in heart muscle at the expense of the acid soluble fraction by producing acute anoxia in dogs.

The biological significance of the glycogen extractable with cold 10% TCA and residual fraction remains to be elucidated. Stetten and Stetten, (1951) believe that these glycogen fractions, as reported by Bloom *et al.* (1951), are artifacts arising from improper purification of the extracts. It is difficult to accept this view when a fundamental difference is noted in the proportions of the two glycogen fractions in different muscles of the same rat over a wide variety of experimental conditions. The percent of acid soluble glycogen extracted from leg muscle was always greater than that found in the diaphragm. Also following similar epinephrine treatment, the residual glycogen was always decreased in the diaphragm but never in the leg muscle. In addition, Merrick and Meyer (1954) report a species difference in the percent TCA soluble glycogen of heart muscle ranging from 100% in goldfish to 19% in mice.

It is conceivable that some of the glycogen in muscle is bound to the protein in such a way that when the protein is precipitated with cold TCA, some of the glycogen is unable to go into solution. Differences in the protein-glycogen binding power in the leg muscle and diaphragm might explain the differences observed between these muscles. According to recent work, all of the glycogen can be removed from muscles ground in a 5% TCA solution and boiled for 15 minutes, the amount obtained being similar to that extracted by hot alkali (Kemp and Van Heijningen, 1954). This posed the question whether a hot TCA extract would permit the recovery of any glycogen, precipitable by alcohol, from these muscles or whether all the glycogen would be hydrolyzed during this extraction procedure. There is no doubt that some of the glycogen would be hydrolyzed. In an experiment on 5 normal rats, designed to determine the amount of alcohol-precipitable glycogen recovered by an extraction with hot TCA, it was observed that of the total glycogen concentration in the leg muscle (379 mg.%), 52 mg. or 13.7% of the total was recovered. In the diaphragm, the total glycogen concentration was 284 mg. %, of which 18 mg. or 6.3% of the total was recovered. Calculation of the residual glycogen would be meaningless in this situation. Thus it is noted that the small amount of glycogen recovered after hot TCA extraction, expressed as a percent of the total glycogen, is twice as high in the leg muscle as in the diaphragm. The percent of TCA soluble glycogen is approximately twice as high in the leg muscle as in the diaphragm in normal rats also, when cold 10% TCA is used. Apparently TCA will remove a certain percentage of the total glycogen

from a given muscle, which is characteristic of the muscle, even though the actual amounts of glycogen recovered may vary with the procedure used.

SUMMARY

Glycogen was determined in a leg muscle (rectus femoris) and diaphragm of rats as (1) the total or hot KOH extractable glycogen (2) a fraction soluble in cold 10% trichloroacetic acid (3) a "residual" fraction calculated by difference of the other two. Characteristically, there was always a higher percentage of TCA soluble glycogen in this leg muscle, compared to the diaphragm, even when the total glycogen was varied by hormonal means. Epinephrine decreased the TCA soluble glycogen but not the residual glycogen in the leg muscle of normal and hypophysectomized rats. Both of these fractions were decreased in the diaphragm under similar conditions.

Changes in these glycogen fractions were studied in the muscles following hypophysectomy and the injection of cortisone and growth hormone. Attempts to explain the interaction of hormones affecting muscle glycogen based on a conversion of the "labile" TCA soluble fraction to the more stable "residual" fraction, were not entirely successful.

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NUTRITIONAL AND HORMONAL INFLUENCES UPON REPRODUCTIVE MATURATION, ORGAN WEIGHTS AND HISTOCHEMISTRY OF THE IMMATURE MALE RAT[1]

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EFFECTS of various types of dietary restriction upon the reproductive system have been investigated. These studies include chronic and acute starvation, caloric restriction, and vitamin and mineral deficiencies (Ershoff, 1952). Of equal importance in reproductive physiology is the availability of more specific food elements such as proteins. Results obtained by Evans and Nelson (1952) and Nelson and Evans (1953) illustrate the deleterious effect of protein depletion during the embryonic period. These same authors (Nelson and Evans, 1954) have shown, however, that pregnancy can be maintained hormonally in the the absence of dietary protein. Protein depletion during the last trimester of pregnancy apparently has little effect on the newborn (Campbell, Innes and Kosterlitz, 1953).

Weight losses of accessory sex organs (Samuels, 1950; Aschkenasy, 1954) and an obvious resistance to loss of testis weight (Leathem and Glasser, 1953) have been noted in adult rats fed protein-free diets. However, maturation of testis and accessories is prevented in immature rats and mice fed protein-free diets (Howell, 1950; Yamamoto and Chow, 1950; Leathem and DeFeo, 1952; Horn, 1953).

The value of dietary protein and hormones in the recovery of adult rats from a protein-free regimen, has been studied (Aschkenasy and Dray, 1953). However, the prevention of reproductive maturation in immature rats by a protein-free diet and the recovery potential when refed protein has not been considered. Autopsy data of organs other than those of the reproductive system, and the histochemistry of the testes, liver, and adrenal are also presented.

MATERIALS AND METHODS

Male rats of the Long-Evans strain were used when 22–23 days of age. A total of 179 animals weighing 42–49 gm. each were caged, four per cage, in an air-conditioned

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room at a temperature of 74°F. Average daily food intake and body weight changes at 5 day intervals were recorded for each animal throughout the experimental period.

All animals except controls were fed a protein-free diet (Table 1), ad libitum, for a "depletion period" of 30 days. Subsequently the rats were divided into groups of 8–15 animals (Groups II and III consisted of 21 and 30 rats respectively) and maintained on diets containing various levels of casein (Table 1) for a "recovery period." Group I was killed at the termination of the "depletion period." Groups II and III were fed a protein-free diet for 10 and 20 days additionally, and killed. A "recovery" diet of 6% casein was supplied to Groups IV, V and VI for periods of 10, 20 and 30 days, in that order. Recovery periods of 10, 20 and 30 days were studied in Groups VII, VIII and IX fed 18% casein.

Following the "depletion period," Groups X, XI and XII were fed an 18% casein diet for 20 days. Each group received 5 gamma of DL thyroxine[3] subcutaneously, once daily, for the entire 20 day refeeding period (Group X); for the first 10 days (Group XI) or for the last 10 days of the 20-day period (Group XII).

Control animals were killed at 22 days of age (Control I), and after 30 days (Control II) and 60 days (Control III) of feeding 18% casein ad libitum.

TABLE 1. EXPERIMENTAL DIETS

Constituents	18% casein diet	6% casein diet	Protein free diet
(in grams)			
S.M.A. Casein	180	60	0
Dextrose	220	340	400
Dextrin	257	257	257
Cellulo Flour	20	20	20
Wesson Salt Mixture	40	40	40
Crisco	250	250	250
Corn Oil	20	20	20
Choline Chloride	2	2	2

To each 100 grams of the above diets were added the following synthetic vitamins: Alpha Tocopherol, 4.0 mgm.; Vitamin A, 900 U.S.P. units; Vitamin D, 180 U.S.P. units; 2-methyl-1,4 naphthquinone diacetate, 1.0 mgm., Thiamine Hydrochloride, 0.8 mgm.; Riboflavin, 1.6 mgm.; Pyridoxine Hydrochloride, 0.8 mgm.; Niacin, 4.0 mgm.; Calcium Pantothenate, 4.4 mgm.; Para-aminobenzoic Acid, 4.0 mgm.; Inositol 21.6 mgm.

At the end of the experimental period, surviving animals were killed with illuminating gas, and the following organs were weighed; testes, seminal vesicles, ventral prostate, spleen, liver, adrenals, kidney, heart, thymus, thyroid, and pituitary. Testes, liver and adrenals were prepared for histological study.

Tissues fixed in Bouin's and sectioned at 10 micra were stained with Harris' hematoxylin and eosin. Those tissues to be studied for fat were fixed in 10% neutral formalin, embedded in 10% gelatin, (testes were embedded in 25% gelatin) sectioned at 15 μ on the freezing microtome, stained with Herxheimer's solution (sudan IV) (Cowdry, 1948), sudan black B (Baker, 1944), Nile blue sulfate (Cowdry, 1948), and the Schultz modification (Schultz, 1924) of the Liebermann-Burchard method. Glycogen was stained by the McManus technique (McManus, 1948; Lillie, 1950), in portions of liver fixed in picro-alcohol-formol (PAF) solution and sectioned at 7μ. The presence of glycogen was confirmed in each case by control sections digested in saliva or diastase for 30 minutes prior to staining. Desoxyribonucleic acid (DNA) was detected by a positive reaction

[3] DL thyroxine was generously supplied by E. R. Squibb and Sons, New Brunswick, New Jersey.

with Schiff's reagent in pieces of testis fixed in a saturated mercuric chloride solution (Feulgen and Rossenbeck, 1924).

RESULTS

Growth, Survival and Food Consumption. Rats on a protein-free regimen not only failed to grow but lost 25% in body weight during the 30-day period (Table 2). Animals on the 0% casein diet for longer than 30 days (Groups II and III) exhibited somewhat greater weight losses and markedly curtailed survival (81-83% fatality).

Following 30 days of protein depletion a refeeding period of similar duration was studied using 6% and 18% casein diets. A 6% casein diet for

TABLE 2. INFLUENCE OF VARIOUS LEVELS OF DIETARY PROTEIN AND THYROXINE UPON SURVIVAL GROWTH AND THE RECOVERY OF ACTUAL REPRODUCTIVE ORGAN WEIGHTS IN IMMATURE MALE RATS PREVIOUSLY FED A PROTEIN-FREE DIET FOR 30 DAYS

Group number	Recovery period		Body weight (surviving rats)[1]				Testis mg.	Seminal vesicles, mg.	Ventral prostate, mg.
	% casein	Days	Start	30 days portein-free	End of recovery	Change			
Control I	(22 days of age)		45 (8)	— —	— —	—	329± 8[2]	8.6± 0.1	7.1± 0.2
I	—	—	43 (8)	32 (8)	— —	−11	103± 7	5.1± 0.1	2.4± 0.2
II	0	10	44 (21)	36 (17)	29 (4)	−15	63± 3	4.7± 0.3	—
III	0	20	49 (30)	38 (22)	33 (5)	−16	77± 5	5.7± 0.2	—
IV	6	10	42 (10)	29 (8)	33 (8)	− 9	118± 12	6.0± 0.3	4.1± 1.3
V	6	20	44 (12)	29 (9)	40 (9)	− 4	197± 4	5.6± 0.3	3.4± 0.9
VI	6	20	46 (8)	31 (8)	48 (8)	2	355± 14	7.4± 0.5	5.1± 0.6
VII	18	10	44 (11)	34 (10)	77 (10)	33	414± 38	15.7± 0.4	10.6± 1.5
VIII	18	20	44 (12)	33 (8)	110 (8)	66	780± 34	22.7± 3.0	16.8± 3.6
IX	18	30	45 (15)	30 (10)	148 (9)	103	1143±116	199.6±12.3	64.8±17.6
			Thyroxine administered days 1 to 20						
X	18	20	44 (12)	32 (10)	129 (9)	85	1151± 64	45.8± 9.2	32.4± 0.9
			Thyroxine administered days 1 to 10						
IX	18	20	42 (14)	30 (8)	101 (8)	59	695± 16	18.9± 4.2	11.9± 1.4
			Thyroxine administered days 10 to 20						
XII	18	20	44 (12)	33 (11)	108 (11)	64	775± 19	23.1± 7.4	17.3± 1.6
Control II[3]			43 (8)	179 (8)	— —	136	1237± 91	433.3±22.6	161.6±11.3
Control III[3]			41 (8)	168 (8)	269 (8)	228	2060±113	684.0±31.7	245.4±19.4

[1] Body weights are given in grams. The number of surviving animals at each phase of the experiment is enclosed in parentheses.

[2] Standard error = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[3] Control Groups II and III were fed an 18% casein diet for 30 and 60 days, respectively, and killed.

30 days (Group VI) permitted a return to starting body weight, whereas an 18% casein diet resulted in a gain of 103 gm. above initial body weight (Group IX). Although body weights of animals fed 18% casein for 30 days following protein depletion were but 54% of those recorded for control animals, both of these groups gained roughly 100 gm. during the last 30-day period.

The possible protein anabolic effects of thyroxine were tested in rats which, following protein depletion, received 5 gamma of thyroxine, daily, concomitantly with an 18% casein diet. The gain in body weight was 30% greater in thyroxine-treated rats than in untreated rats fed the same diet

for an identical period (Group VIII). Shorter periods of thyroxine treatment were without effect (Group XI and XII).

Food intake was influenced by the diet fed. In the absence of dietary casein, 3.3 gm. of food were eaten daily for 30 days. Feeding a 6% casein diet during recovery from protein depletion did not improve daily food intake. The 18% casein diet increased food intake to 6.7 gm. daily but was not enhanced by thyroxine.

Organ Weights and Histology

Testis. An actual loss of testis weight occurred in all rats during the 30-day protein-free regimen. Testis weight to body weight increased, however. Refeeding an 18% casein diet resulted in a 1040 mg. testis weight gain

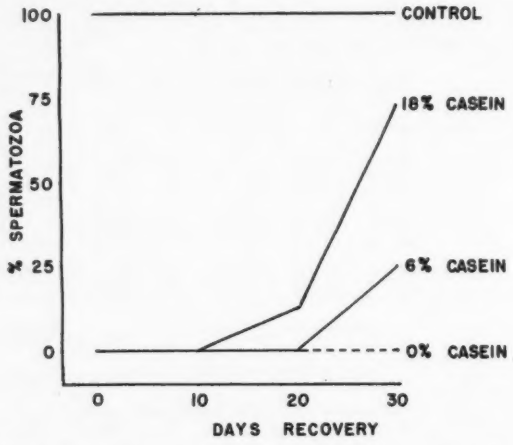

FIG. 1. Influence of dietary protein on spermatogensis previously retarded by a protein-free diet fed to immature rats for 30 days.

in 30 days, but the gonad weight was still 50% less than that of a non-depleted rat. In contrast, 6% casein refeeding permitted only a 252 mg. testis weight gain in 30 days. Thyroxine, administered daily throughout a 20-day recovery period, augmented testis weight grain in rats fed 18% casein. Nevertheless, the recovery in all instances was incomplete (Table 2).

Testes of the 22-23 day old rats contained secondary spermatocytes. The testes of control animals, fed an 18% casein diet for a period of 30 days, exhibited mature spermatozoa whereas rats fed a protein-free diet for 30 days showed a reduction in the size of the seminiferous tubules and congestion of sloughed germinal elements; primary spermatocytes were the most advanced cells. When the protein fast was continued beyond 30 days, tubular size was further reduced and congestion increased.

Following a 30-day protein fast, a 6% casein diet permitted 25% of the animals to form spermatozoa in 30 days (Fig. 1). An 18% casein diet, however, aided spermatogenesis to a much greater degree; 70% of the animals produced spermatozoa (Fig. 1). Thyroxine augmented the rate of spermatogenic recovery in protein-depleted rats being refed on 18% casein. After 20 days of thyroxine treatment, spermotozoa were present in the tubules of 88% of the testes (Group X) as opposed to a 12% recovery in untreated rats (Group VIII). When thyroxine was injected for the first 10 days of the 20 day recovery period, 37% of the animals had spermatozoa, whereas injections during the last 10 days permitted 27% of the animals to develop spermatozoa. Thus, some spermatogenic stimulation was observed with all patterns of thyroxine treatment (Fig. 2).

Control testes stained with sudan black B revealed a characteristic lipid

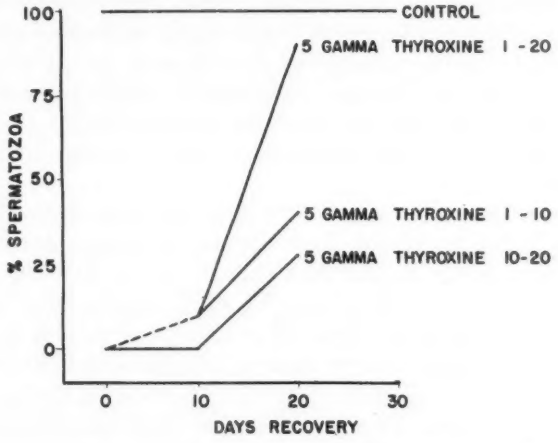

FIG. 2. Enhancement of spermatozoa formation with thyroxine during 18% casein feeding of prior protein-depleted immature rats.

configuration. In tubules containing spermatozoa, the lipid was finely particulate and occupied a position adjacent to the spermatozoa and spermatids. Tubules in the resting phase and lacking spermatozoa contained globular lipid which formed an annular, sudanophilic band at the basement membrane. This lipid appeared to be contained in the Sertoli cells as well as in many of the spermatogonia. Interstitial tissue was free of lipid in all instances.

The testes of rats fed a protein-free diet presented globular sudanophilic bands similar to, but of greater magnitude than those observed in normal tubules in the resting phase. The sudanophilic band was even more extensive in animals which survived for periods longer than 30 days on a protein-free diet.

Histologically demonstrable neutral fats, unsaturated glycerides and cholesterol esters were absent in rat testes.

Desoxyribonucleic acid (Feulgen reaction) in normal rat testes was concentrated in the heads of spermatozoa and the nuclei of spermatogonia and primary spermatocytes. The nuclei of secondary spermatocytes and spermatids were, by comparison, less concentrated. This distribution and intensity of staining of the material was affected by the deletion of dietary protein only in so far as controlling what cells of the spermatogenic line were present.

Accessory Sex Organs. Atrophy of the seminal vesicles from 8.6 to 5.1 mg. followed 30 days of protein depletion and a more pronounced weight decrease occurred in the ventral prostate. The ventral prostate was not dissectable when the protein-free regimen was extended beyond 30 days. Feeding 6% casein during the recovery period failed to increase accessory organ weights. Recovery on an 18% casein diet permitted only a modest accessory weight increase during the first 20 days, but a marked weight gain occurred after the 20th day. Nevertheless seminal vesicle weights of 119.6 mg. were considerably less than the 684 mg. weight of these organs in fully fed rats. A modest enhancement of accessory organ weight gain was obtained with thyroxine.

Liver, Adrenal and Other Organs. Weight changes of these organs are tabulated in Table 3. Actual liver weight decreased during a 30-day protein-depletion period to less than that of the 22 day old rat. Weight recovery reflected the level of protein fed. Liver lipid was not detectable in 22 day old rats on stock diet (Fox Chow), but was present in rats fed an 18% casein diet for either 30 or 60 days as fine particulate droplets around the central vein. Lipid was dramatically increased in the livers of rats fed a protein-free diet. Normal liver lipid patterns were restored more quickly with 18% casein than with 6% casein diets. Protein fasting increased liver glycogen with no apparent decrease occurring during the 30-day recovery period when either 6% or 18% casein was fed.

Adrenal atrophy occurred during the 30 days when rats ate a protein-free diet. Regardless of the recovery regimen, only partial restoration of normal adrenal weight occurred. In general, protein starvation caused an increase in adrenal lipid. Incomplete recovery of normal neutral fat distribution resulted with both 6% and 18% casein diets for a full 30-day recovery period.

Actual weights of the pituitary, thyroid, kidney, heart, thymus, and spleen revealed atrophic changes during protein starvation, However, organ weight to body weight ratios showed hypertrophy in all but thymus, which atrophied both actually and relatively. Weight gain of these organs, following the 30-day period of depletion, depended directly upon the amount of dietary protein provided and the duration of the refeeding interval.

TABLE 3. INFLUENCE OF VARIOUS LEVELS OF DIETARY PROTEIN AND THYROXINE UPON SURVIVAL, GROWTH, AND THE RECOVERY OF ACTUAL ORGAN WEIGHTS[1] IN IMMATURE MALE RATS PREVIOUSLY FED A PROTEIN-FREE DIET FOR 30 DAYS

Group number	Recovery diet		Body weight	Liver	Adrenal	Pituitary	Thyroid	Heart	Spleen	Thymus	Kidney
	% Casein	Days									
Control I	(22 days of age)		45	3,594 ± 73[2]	93. ±0.3	3.7 ±0.1	5.8 ±0.2	314 ±14	350 ± 52	93.3 ±14.6	619 ± 42
I	—	—	32	1,117 ± 122	5.8 ±0.3	2.1 ±0.3	3.8 ±0.2	158 ± 8	72 ± 24	7.6 ± 0.5	379 ± 33
II	0	10	29	816 ± 94	3.7 ±0.2	1.2 ±0.1	2.1 ±0.2	151 ±12	33 ± 9	—	351 ± 20
III	0	20	33	1,434 ± 86	3.3 ±0.4	1.6 ±0.2	3.0 ±0.3	191 ± 8	51 ± 7	—	480 ± 31
VI	6	10	33	1,628 ± 137	8.0 ±0.3	2.0 ±0.3	3.9 ±0.3	190 ± 8	127 ± 31	14.4 ± 0.8	483 ± 29
V	6	20	40	2,098 ± 132	6.6 ±0.3	2.3 ±0.1	3.5 ±0.3	227 ±12	161 ± 28	26.4 ± 3.2	518 ± 25
VI	6	30	48	2,385 ± 98	7.8 ±0.3	2.6 ±0.3	3.8 ±0.2	243 ±18	242 ± 83	44.2 ± 7.6	541 ± 26
VII	18	10	77	4,284 ± 197	11.4 ±0.3	3.3 ±0.2	6.5 ±0.4	345 ±18	578 ±124	121.9 ±13.6	903 ± 16
VIII	18	20	110	5,187 ± 138	14.7 ±0.5	4.6 ±0.2	6.9 ±0.5	427 ±19	894 ±113	229.2 ±31.5	1,129 ± 42
IX	18	30	148	6,823 ± 202	14.4 ±0.4	6.2 ±0.3	9.3 ±0.4	510 ±24	544 ± 17	247.8 ±19.7	1,378 ± 74
				Thyroxine administered days 1 to 20							
X	18	20	129	5,787 ± 212	12.8 ±0.3	4.6 ±0.2	5.4 ±0.3	480 ±18	732 ± 11	293.8 ±12.1	1,263 ± 33
				Thyroxine administered days 1 to 10							
XI	18	20	101	5,164 ± 236	11.1 ±0.5	4.1 ±0.2	6.2 ±0.3	448 ±11	568 ± 10	134.0 ± 8.8	1,326 ± 26
				Thyroxine administered days 10 to 20							
XII	18	20	108	5,306 ± 298	12.3 ±0.3	4.4 ±0.4	5.8 ±0.3	420 ±25	753 ± 27	166.7 ±15.3	1,100 ± 19
Control II[3]			168	7,202 ± 451	14.9 ±0.5	6.1 ±0.3	9.7 ±0.4	569 ±22	629 ± 41	277.8 ± 7.4	1,429 ± 27
Control III[3]			269	10,315 ±1,036	20.1 ±0.2	6.7 ±0.2	15.6 ±0.9	737 ±71	866 ± 30	341.4 ± 8.9	2,136 ±140

[1] All organ weights are given in milligrams; body weights in grams.

[2] Standard error $= \sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[3] Control Groups II and III were fed an 18% casein diet for 30 and 60 days, respectively, and killed.

DISCUSSION

Reproductive Organs

Adult infertility might be reflection of prepuberal disturbances. These disturbances may be induced by abnormal nutrition or hormonal activity during the period of seminiferous tubule development. Altered nutrition, such as chronic starvation, will prevent maturation of the gonad in immature rats and will abolish active gonadal function in the adult (Mulinos and Pomerantz, 1940). Diets lacking only protein, however, are less effective in abolishing spermatogenesis in adult rats (Leathem, 1954) than is starvation, but when fed to the actively growing rat, maturation of the testis is completely inhibited. In young rats, a diet containing 6% casein was not adequate for body growth, but did permit formation of some spermatozoa (Guilbert and Goss, 1932; Horn, 1953). Thus, the reproductive system may gain special consideration in protein allotments when supplies are limited.

Dietary retardation of the testis favors the accumulation of lipid in the spermatogonia and primary spermatocytes as well as in Sertoli cells, an effect also noted in the adult rat (Leathem, 1954). Previously, Lynch and Scott (1951) reported an abundance of lipid only in the Sertoli cells following hypophysectomy. The accumulation of lipid in the inactive testis may imply either non-utilization or an abnormal assimilation.

Estimation of changes in ribonucleic acid (RNA) and desoxyribonucleic acid (DNA) concentration, histochemically, in testes of rats subjected to protein starvation, closely resembled the changes observed by McEnery and Nelson (1953) in hypophysectomized rats. Both RNA and DNA were influenced by the spermatogenic activity of the seminiferous tubule. Testis nitrogen in adult rats was only slightly decreased by a protein free diet but was sharply depressed by hypophysectomy (Leathem, 1954).

While it is apparent that testis maturation is prevented by many nutritional deficiencies and hormonal imbalances, it is the effect of these factors on the potential for recovery of this organ and its accessories which has been least studied. Ninety-five per cent of the rats initially fed a diet containing 6% casein for 30 days have testes containing spermatozoa after only 10 days of feeding an 18% casein recovery diet (Horn, 1953). On the other hand, only 25% of the rats initially fed a protein-free diet for 30 days reveal spermatozoa after 30 days refeeding a diet containing 18% casein. Therefore, the current studies do not indicate that permanent reproductive damage is invoked by the experimental design used.

Since starvation reduces the level of circulating thyrotrophin (D'Angelo, 1951), protein depletion may also decrease thyroid function, suggesting that thyroxine might aid the return of full testis function during protein repletion. DL-thyroxine clearly enhanced testis maturation in comparison to dietary effects alone. The immature rat normally operates at maximum

thyroid activity and supplemental thyroxine decreases the animal's response to gonadotrophins. In contrast, the mouse, rabbit and ram (Maqsood and Reineke, 1950; Maqsood, 1951b), testis maturation and responsiveness can normally be stimulated with physiological dosages of thyroxine. These species are suggested as suboptimal in normal thyroid function (Koger, Hurst and Turner, 1942). Possibly then, the rat thyroid is subnormal because of protein inadequacies and apparently recovers slowly on adequate diets, so that supplemental thyroxine is beneficial. In adult rats, however, thyroxine did not influence testis weight when 7% casein was fed following protein deprivation (Aschkenasy and Dray, 1953) and did not decrease testis weight when given to rats fed protein-free diets (Aschkenasy, 1954).

OTHER ORGANS

Weight losses of other organs (liver, adrenal, pituitary, thyroid, heart, spleen, thymus and kidney) seen in immature rats starved of protein for a period of 30 days, are similar to those losses reported for mature rats (Addis, Poo and Lew, 1936; Kosterlitz and Campbell, 1945–46; Aschkenasy, 1954). At both periods of life, organ weight loss is roughly proportional to body weight loss except for the testis, and the pituitary; these organs lost proportionally less during the period of protein starvation than the carcass, per se.

An increase in liver lipid following protein depletion is not surprising and is common to many influences exerted upon the animal (Patek, Plough and Bevans, 1954). The increase in liver glycogen during the protein fast is particularly noteworthy and without satisfactory explanation (Kosterlitz, 1947).

The increase in adrenal lipid during protein starvation might well indicate a decrease in hormone production (Bergner and Deane, 1948; Sayers and Sayers, 1948; Ducommun and Mach, 1949) as does adrenal atrophy. The partial return of adrenal lipid upon refeeding might likewise indicate return of secretory activity. The effect of protein starvation is similar to that of chronic inanition in that the adrenal is atrophied in both instances, but is wholly unlike the effect of acute starvation in causing adrenal hypertrophy.

SUMMARY

Immature male rats lost weight when fed a protein free diet and after 30 days, survival was markedly curtailed. Testis maturation was prevented by omission of dietary protein and tubular lipid was increased. Permanent testicular damage was apparently not invoked by delaying maturation, for spermatozoa did develop in the testis of rats returned to 18% casein diets. Thyroxine augumented the recovery of testis function fostered by nutrition. Testis maturation was not related to body weight recovery.

Accessory sex organ weights were only partially recovered after 30 days refeeding on an 18% casein diet and indicate that spermatogenic maturation and androgen release are not operating at the same level.

Lipid and glycogen concentration were increased in the livers of protein fasted rats with partial disappearance of the increased accumulation of both following refeeding. Likewise, lipid was increased in the adrenals of immature protein fasted rats. Partial reduction of adrenal lipid was observed with refeeding 18% casein diets.

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VARIATIONS IN ADRENAL ASCORBIC ACID CONCENTRATION OF THE HAMSTER DURING THE PRE-HIBERNATORY PERIOD

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INTRODUCTION

THE Golden Hamster, as an example of a "deep hibernator" (Lyman, 1948), undergoes a striking decline in the body temperature and metabolic rate on entering hibernation.

The response of the rat to a lowering of the environmental temperature induced what has been termed "cold stress" (Selye, 1946). This condition in the rat is characterized by depletion of the adrenal ascorbic acid concentration, which, in turn, is considered indicative of increased ACTH activity (Sayers and Sayers, 1948).

The purpose of this investigation was to study the adrenal cortical response of a hibernator, such as the Golden Hamster, from the time of entering the cold until complete hibernation. Variations in the adrenal ascorbic acid concentration were used as indices of adrenocortical activity.

MATERIALS AND METHODS

Hibernation was artificially induced by housing the animals in individual cages and placing them in the cold room at 2° C. Each animal was placed in a cage, together with hay for nesting purposes and adequate food for the pre-hibernatory period. The cage was then placed on a tray containing sawdust. This arrangement insured adequate sanitary conditions during the period of the experiment, and obviated the necessity of disturbing the animals for cleaning purposes once the animals were under experimental conditions. Normal control animals were kept in the animal house at 26 to 29° C. The experimental animals were young male hamsters which were put in the cold room for varying periods: ½ hour (4 animals); 3 hours (4 animals); 5 hours (6 animals); 41 hours (4 animals); 138 hours (4 animals); and finally, animals which had undergone hibernation (8 animals). Control animals (10) were those taken from the animal house at a temperature of 26 to 29° C. On killing the animals, the adrenals were removed, cleaned of adhering fat and weighed on a torsion balance as quickly as possible. The body weight was also recorded.

The chemical ascorbic acid content of the adrenals was determined according to the method of Roe and Keuthner (1943) and the colorimetric estimations were made on a Hilger Spekker Absorptiometer.

It became evident as the work progressed that cold might not constitute a "stress" to the hamster as it does to the rat. Therefore, it was decided to try another recognized

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form of stress, histamine, on the hamster. Unfortunately, it was only possible to run estimations on four young mature male animals. These were injected with 1 mg./100 gm. body weight of histamine acid phosphate and killed one hour later. The adrenal glands were then removed, weighed and assayed for ascorbic acid concentration.

RESULTS

The normal mean ascorbic acid concentration of hamster adrenals was 309.9 ± 17.8 mg./100 gm. of adrenal tissue. These results were obtained from 10 animals kept at 26°–29° C.

Animals which had been exposed to the cold (2° C.) for ½ hour showed an adrenal ascorbic acid concentration of 287.5 ± 15.2 mg.%, a value slightly less than the normal figure, but the difference was not statistically significant ($P < 0.5$). During the next 3 hours in the cold there was a steady rise in the mean ascorbic acid concentration, the mean value after 3 hours being 468.5 ± 29.1 mg.% for the six animals so treated. This rise was signifi-

TABLE 1. BODY AND ADRENAL WEIGHT, AND ADRENAL ASCORBIC ACID CONCENTRATION OF HAMSTERS DURING THR PRE-HIBERNATORY PERIOD AND HIBERNATION

Hours at 2° C.	No. of animals	Avg. body wt., gm.	Adrenal wt., mg.	a^1 / B	Ascorbic acid, conc. mg./100 gm. tissue	Fisher's P value
0	10	88.6 ± 2.1^2	15.8 ± 0.4	17.8 ± 0.5	309.9 ± 17.8	—
0.5	4	65.0 ± 0.3	14.0 ± 0.3	21.6 ± 0.4	287.5 ± 15.2	$> .3, < .5$
3	6	90.3 ± 2.0	17.2 ± 0.3	19.2 ± 0.3	468.7 ± 29.1	$> .05, < .1$
5	6	100.0 ± 4.2	23.7 ± 1.0	23.7 ± 1.0	369.5 ± 19.5	$> .001, < .01$
41	4	76.3 ± 3.1	20.8 ± 0.4	27.3 ± 0.8	336.2 ± 19.0	$> .2, < .3$
138	4	72.7 ± 3.5	20.6 ± 1.1	28.3 ± 0.7	302.2 ± 9.6	$> .3, < .5$
H[3]	8	53.6 ± 2.0	15.1 ± 0.6	27.9 ± 0.6	306.9 ± 14.0	$> .5$

[1] $\frac{a}{B} = \frac{\text{Adrenal weight mg.}}{\text{Body weight gm.}} \times 100.$

[2] Standard error of mean.

[3] Hibernating.

cant compared to the normal values ($P < 0.1$). Two hours later, that is after a total of 5 hours at 2° C, the ascorbic acid concentration had fallen to 369.5 ± 19.5 mg.%; this was still significantly higher than the control value ($P < .01$). From this point until the animals entered hibernation there appeared to be a steady fall in the ascorbic acid concentration. After 41 hours in the cold the concentration was 336.2 ± 19.0 mg.% and after 138 hours the value was found to be 302.2 ± 7.6 mg.%. The difference between these values and the normal was also decreasingly significant ($P < 0.3$ and < 0.5 respectively). The adrenals of hibernating hamsters contained 306.9 ± 14.0 mg.% of ascorbic acid; this concentration was not significantly different from the normal control value ($P < .5$).

The changes in adrenal ascorbic acid are summarized in Table 1 and expressed graphically in Figure 1.

The effect of histamine as a stressing agent

Hamsters injected with 1 mg./100 gm. body weight of histamine acid phosphate and killed 1 hour later demonstrated that the hamster reacted

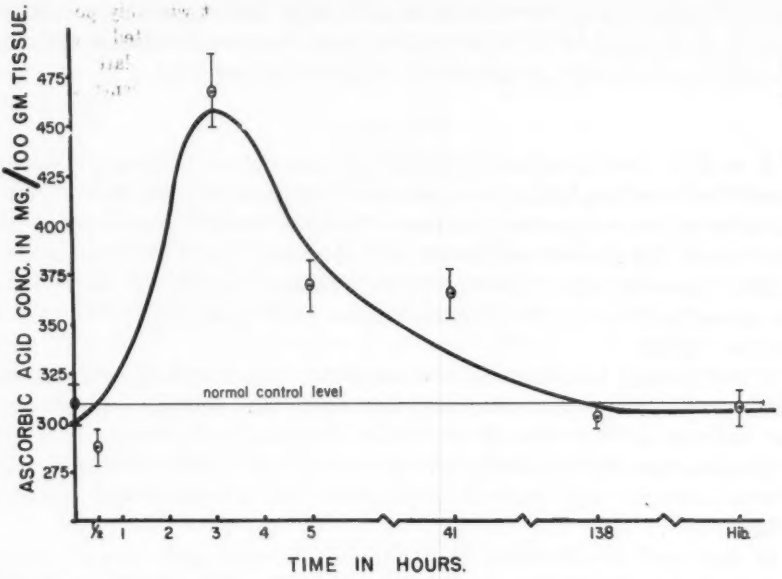

FIG. 1. Variations in the adrenal ascorbic acid concentration of the hamster during the pre-hibernatory period. The normal control level was estimated from animals kept in an environment at 26 to 29° C.

in a similar manner to the rat when subjected to stress other than cooling. The results of the adrenal ascorbic acid assay from hamsters subjected to histamine stress are summarized in Table 2. The mean adrenal ascorbic acid concentration from histamine stressed animals showed a 22.8% depletion when compared with the normal control value. This depletion is significant ($P < 0.001$).

Adrenal weights

The adrenal weights of each set of animals were expressed as a mean adrenal weight in mg./100 gm. body weight (Table 1). The normal figure for control group was 17.8 ± 0.4 mg.%. During the pre-hibernatory period there was a steady increase in the a/B ratio which was maintained for 41 hours in the cold; after this time there was no significant increase in

TABLE 2. EFFECT OF ONE SUBCUTANEOUS INJECTION OF HISTAMINE ACID PHOSPHATE 1 HOUR BEFORE ASSAY OF ADRENAL ASCORBIC ACID CONCENTRATION

No. of animals	Body wt., gm.	Dose mg.	Ascorbic acid/100 gm. tissue	% depletion of normal
10	88.6 ± 2.1[1]	nil	309.9 ± 17.8	0
4	80.7 ± 4.2	0.8	239.3 ± 11.7	22.8

[1] Standard error of mean.

the a/B values. Any increase in the a/B ratio from 41 hours onwards was due to decrease in body weight rather than increase in adrenal weight, as the animals were kept progressively longer in the cold.

DISCUSSION

It is clear that the adrenal weights and adrenal ascorbic acid concentrations of hibernating hamsters do not differ significantly from those of normal animals kept in a warm environment. Also, the initial response of the hamster to cold is in distinct contrast to that of the rat, where cold is an acknowledged "stressing agent" (Selye, 1946). The latter animal is characterized by an early decline in the adrenal ascorbic acid concentration (Sayers and Sayers, 1948).

If the adrenal ascorbic acid concentration does fluctuate concomitantly with adrenocortical secretion, then this would lead to the assumption that the activity of the adrenal cortex in hibernating hamsters is normal. Similarly, since the initial response of the hamster to cold is one of increased adrenal ascorbic acid content, presumably the adrenocortical activity is lessened.

It may well be, however, that adrenal ascorbic acid content is not a reliable index of adrenocortical function since the differentiation of ACTH into "adrenal weight increasing" and "ascorbic acid depleting" fractions has been demonstrated (Young 1952; Li 1952).

Assuming the adrenal ascorbic acid level to be a reflection of continued active secretion in the hibernant, then this must necessarily be concerned with the particular metabolic activities associated with hibernation. Lyman and Leduc (1953) have shown that the blood sugar level of hibernating hamsters is slightly higher than that of control animals. Furthermore, they state that there is no difference between the glycogen concentration of liver and skeletal nuscle in hibernating and non-hibernating animals. The near-normalcy in the hibernant of these aspects of carbohydrate metabolism might depend, in part, on continued adrenocortical secretion. Studies of hibernation in the ground squirrel by Vidovic and Popovic (1954) indicate that this animal is unable to re-enter a state of hibernation after adrenalectomy. Also, the animals did not continue to hibernate after being adrenalectomized during the hypothermic condition. Therefore, if the metabolism of these two "deep hibernators" is similar, a continued secretion of adrenal hormones would appear necessary for the maintenance of hibernation in both instances. It is relevant in this respect to find that the hamster enters hibernation with very little white fat, although it possesses brown fat. Interestingly enough, brown fat has been shown to be under adrenal steroid control (Fawcett and Chester Jones, 1949; Bole *et al.*, 1951).

In agreement with Deane and Lyman (1954), we may conclude that cold

does not constitute a stress to the hamster. Also, we agree with the above workers' findings that there is no involution of the adrenal cortex of the hamster prior to hibernation. Indeed, the adrenal cortex would seem to be quite active.

SUMMARY

The adrenal ascorbic acid concentration of the Golden Hamster (Mesocricetus auratus), under prehibernatory and hibernatory conditions, was estimated according to the method of Roe and Keuthner (1943).

There was no significant difference between the adrenal ascorbic acid concentration of the control group (309.9 ± 17.8 mg. per 100 gm. adrenal tissue) and the group of hibernating animals (306.9 ± 14.0 mg.%)

On placing the animals in a cold environment (2° C.) there was a rise in the adrenal ascorbic acid concentration. This rise continued for about 5 hours, after there was a steady decline until the animals hibernated.

The possibility that the adrenal cortex of the hibernating hamster is actively secreting cortical hormones is discussed.

Acknowledgment

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EFFECT OF THYROID STATUS ON SUCCINATE OXIDATION BY VARIOUS TISSUES OF THE RAT[1,2]

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IT HAS been known since the work of Dye and Maughan (1929) that the succinoxidase activity of skeletal muscle was diminished by thyroidectomy. Several groups of workers have shown liver succinoxidase to be sensitive to the absence or presence in excess of the thyroid hormone in the same direction as the energy metabolism (cf. Barker, 1951). In contrast, brain succinoxidase was reported by Rossiter (1940) to be unchanged by administration *in vivo* of desiccated thyroid, just as the oxygen consumption of this tissue was unaltered in experimental hyperthyroidism. Recent work has revealed that other tissues, like brain, exhibit no variation in energy metabolism under the influence of thyroxine or thyroidectomy (Barker and Schwartz, 1953). In order to determine how consistent the correlation between changes in oxygen consumption and succinoxidase activity would be, the present series of experiments was undertaken, employing tissues with and without oxygen consumption responses to thyroid status.

METHODS

The rate of oxidation of succinate was studied using two procedures: the Schneider and Potter manometric, involving the series of enzymes from succinic dehydrogenase through the cytochromes, measured by oxygen consumption (cf. Potter, 1949), and the "formazan," depending upon colorimetric determination of triphenyl formazan formed by reduction of 2,3,5-triphenyl-tetrazolium chloride in the presence of succinate (Barker *et al.*, unpublished). It is reasonably clear that the latter procedure involves flavoprotein as well as succinic dehydrogenase (Brodie and Gots, 1952), although it has been demonstrated that the amount of proton and electron movement in the presence of the dye acceptor is only about 2 to 5% of that calculated from the oxygen consumption figures obtained by the manometric procedure. It does not seem possible at present to evaluate any more specifically the relationships between these two measurements. Wherever the amounts of tissue available permitted, both techniques were employed for comparative purposes.

Sprague-Dawley rats were used. When the animals were thyroidectomized, at least 4 weeks elapsed after the operation for maximal hypometabolism to develop. To some of the thyroidectomized animals, thyroxine (used as the sodium L-thyroxinate, penta-

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hydrate[3]) was injected, 2 mg. per kg. body weight per day for 4 days and the sacrifice made on the 5th day. Each animal was exsanguinated unde rether anesthesia, and the desired tissues rapidly removed and chilled in Kreb's Ringer solution at 0°. Grinding was accomplished in Potter-Elvehjem glass homogenizers at 0°, using water as the medium.

TABLE 1. COMPARISON OF SUCCINATE OXIDATION IN EUTHYROID, THYROIDECTOMIZED AND HYPERTHYROID RATS

Tissue	Tech.	Euthyroid			Thyroidectomized			Hyperthyroid		
		No.	Mean*	S.D.	No.	Mean	S.D.	No.	Mean	S.D.
Kidney	Man	12	88.8	±12.5	10	76.8	±7.3	12	82.9	± 9.8
	TTC	12	1.86	± 0.12	10	1.68	±0.24	12	1.92	± 0.14
Heart	Man	14	82.3	± 4.2	14	67.7	±2.9	12	84.4	±12.5
	TTC	10	2.69	± 0.22	10	1.65	±0.24	12	2.48	± 0.25
Liver	Man	14	48.7	± 3.4	14	43.8	±5.5	12	56.3	± 8.2
	TTC	11	1.69	± 0.09	13	1.44	±0.08	12	2.23	± 0.14
Saliv. gl.	Man	12	27.1	± 2.9	10	27.0	±1.4	12	28.5	± 0.8
	TTC	12	0.53	± 0.05	10	0.31	±0.06	12	0.60	± 0.05
Diaphr.	Man	10	22.1	± 2.7	14	18.0	±1.9	12	19.9	± 2.5
	TTC	10	0.56	± 0.08	10	0.31	±0.02	11	0.51	± 0.02
Skel. Mus.	Man	10	7.5	± 0.6	10	6.3	±0.6	12	8.0	± 2.1
	TTC	12	0.24	± 0.04	10	0.14	±0.02	12	0.20	± 0.03
Pancreas	Man	6	6.1	(± 2.2)†	6	9.9	±0.9	6	6.6	(± 1.4)†
	TTC	6	0.17	± 0.02	6	0.27	±0.03	6	0.32	± 0.13
Brain	Man	12	13.8	± 1.0	12	14.6	±1.1	12	14.8	± 1.1
	TTC	12	0.31	± 0.005	12	0.31	±0.02	12	0.34	± 0.01
Spleen	Man	12	8.5	± 0.8	12	8.3	±1.6	12	8.5	± 0.8
	TTC	12	0.28	± 0.07	12	0.27	±0.06	12	0.28	± 0.01
Lymph N.	TTC	8	0.20	(± 0.07)†	9	0.18	(±0.05)†	10	0.18	(± 0.09)†
Testis	Man	6	3.5	± 0.4	8	3.2	±0.1	6	3.3	± 0.1
	TTC	6	0.16	± 0.02	7	0.14	±0.02	6	0.15	± 0.01
Sem. Ves.	Man	6	7.2	± 1.5	6	5.0	±0.1	6	5.2	± 1.0
	TTC	6	0.14	± 0.01	6	0.04	±0.02	6	0.04	± 0.02
Uterus	Man	9	4.6	± 0.8	8	2.9	±0.9	8	3.1	± 0.9
Thymus	Man	14	4.3	± 0.9	13	2.3	±0.9	14	2.6	± 1.1
Ovary	Man	8	7.8	± 1.0	8	7.9	±2.0	8	5.8	± 1.4
Prostate	Man	6	6.6	± 1.0	6	6.5	±0.1	6	6.0	± 0.7
	TTC	6	0.14	± 0.03	6	0.10	±0.02	6	0.10	± 0.02

* Succinate oxidation expressed as μl. H_2 transferred per mg. tissue per hr. ± Standard Deviation.
† One value from each of these series was zero and was omitted from the calculations in each instance.

RESULTS AND DISCUSSION

The results with both procedures as calculated to a common denominator of hydrogen transfer, are summarized in Table 1. Each tissue is represented by 6 to 14 experiments in each of the categories of euthyroid, thyroidectomized or hyperthyroid. It is apparent that the same twenty- to fifty-fold difference in activity, previously encountered in tissues of normal rats, was also obtained with those of hypothyroid and hyperthyroid rats. Although all samples were rapidly chilled and handled alike in the cold, as is noted in the table, occasional pancreas and lymph node samples were encountered with practically no activity. Since this effect was shown by

[3] Sodium L-thyroxine was generously furnished by Smith, Kline & French Laboratories and by the Travenol division of Baxter Laboratories.

TABLE 2. CHANGE IN SUCCINATE OXIDATION PRODUCED BY THYROIDECTOMY AND BY THYROXINE INJECTION

Tissue	Tech.	Thyroidectomy		Thyroidect. + Thyroxine	
		%*	*P*	%†	*P*
Kidney	Man	−13.5	<0.02, >0.01	+ 7.9	<0.05, >0.02
	TTC	− 9.7	<0.01	+14.2	<0.01
Heart	Man	−17.7	<0.01	+24.7	<0.01
	TTC	−38.7	<0.01	+50.0	<0.01
Liver	Man	−10.1	<0.01	+28.5	<0.01
	TTC	−14.8	<0.01	+54.9	<0.01
Saliv. Gl.	Man	− 0.4	>0.10	+ 5.6	<0.01
	TTC	−41.5	<0.01	+93.5	<0.01
Diaphr.	Man	−18.6	<0.01	+10.6	<0.01
	TTC	−44.6	<0.01	+64.5	<0.01
Skel. M.	Man	−16.0	<0.01	+27.0	<0.01
	TTC	−41.7	<0.01	+42.9	<0.05, >0.02
Pancreas	Man	+62.3	<0.01	−33.3	<0.01
	TTC	+58.8	<0.01	+18.5	>0.1
Brain	Man	+ 5.8	<0.1, >0.05	+ 1.4	>0.1
	TTC	0.0		+ 9.7	<0.1, >0.05
Spleen	Man	− 2.4	>0.1	+ 2.4	>0.1
	TTC	− 3.6	>0.1	+ 3.7	>0.1
Lymph Nodes	TTC	−10.0	>0.1	0.0	
Testis	Man	− 8.6	<0.01	+ 3.1	>0.1
	TTC	−12.5	<0.05	+ 7.1	>0.1
Sem. Ves.	Man	−30.6	<0.01	+ 4.0	>0.1
	TTC	−75.0	<0.01	+14.7	>0.1
Uterus	Man	−37.0	<0.01	+ 7.9	> 0.1
Thymus	Man	−26.5	<0.01	+13.0	>0.1
Ovary	Man	+ 1.3	>0.1	−26.6	<0.01
Prostate	Man	− 1.5	>0.1	− 7.7	<0.05, >0.02
	TTC	−28.6	>0.1	0.0	

* % thyroidectomized/euthyroid.
† % thyroxine-injected thyroidectomized/thyroidectomized.

both duplicates in either procedure, but not by both procedures in any given instance, it is difficult to explain on the basis of the occasional presence of an unknown inhibitor.

Table 2 contains the percentage changes in succinate oxidations from normal to hypothyroid and hypothyroid to thyroxine-injected thyroidectomized rats. The corresponding P values were obtalned by the t-test.

Liver, heart, kidney, diaphragm, skeletal muscle and salivary gland, all of which display oxygen consumption changes related to the amount of thyroid hormone available, showed similar alterations, for the most part, in succinoxidase levels by either procedure. In every instance, differences

were greater by the colorimetric procedure. At the present time, it is not possible to go further than to mention the possibility that this may indicate a greater effect of thyroxine on dehydrogenase and flavoprotein than on cytochromes.

Pancreas, which also belongs metabolically in the group of tissues responding to thyroid status, displayed the inexplicably bizarre alterations of 60% increases in succinoxidase after thyroidectomy and either no change or a marked drop in activity of this enzyme when thyroxine was injected, depending upon the technique of study.

The remaining tissues, brain, spleen, ovary, uterus, testis, prostate, seminal vesicle, thymus and lymph nodes, are those whose energy turnover is not responsive to thyroxine lack or excess. In contrast to the first group, here the succinoxidase changes either were absent, as with brain, spleen and lymph nodes, or were not consistent. For instance, testis, seminal vesicle, uterus and thymus enzyme activities were significantly lowered by thyroidectomy, but were not raised by hyperthyroidism. Ovarian and prostatic activities were not affected by hypothyroidism but were lowered by thyroxine injection.

Despite some irregularities, outstanding in the specific case of the pancreas, the succinoxidase changes resulting from thyroidectomy or thyroxine injection may be considered to support the concept that the hormone controls metabolic rate through some influence on important enzyme systems, as exemplified by succinoxidase. This theory is further supported by considerable evidence concerning the cytochrome *c* and cytochrome oxidase levels of liver, kidney, heart, skeletal muscle, brain and spleen (cf. Barker, 1951). Those tissues of the animal body which are altered enzymatically consistently show an increased oxidative metabolism. In contrast are those tissues containing enzymes resistant to alteration, even by the same amount of hormone.

The work of Gemmill (1952) and Wiswell *et al.* (1954) was directed toward demonstrating a possible coenzyme role for thyroxine in succinoxidase systems. From this point of view, it might be considered that the hormone was somehow incapable of functioning as a cofactor in those tissues showing no response. Studies are now being carried out in this laboratory on this point.

Barron pointed out several years ago that all tissue enzyme potentialities were greater than their oxygen consumption equivalents (1943). He postulated that this difference was due to control exerted by some unknown physiological inhibitor, but it seems equally reasonable to attribute it to an effect of specific spatial orientations within the cell. By virtue of the peculiar electrostatic effects of the thyroxine configuration, intracellular distribution of enzyme systems might be changed considerably. Even as broad a concept as this cannot explain why thyroxine is not able to produce equivalent enzymatic changes in all tissues. Although evidence (Klitgaard

and Barker, unpublished) shows that thyroxine is present in all tissues of the animal after injection of a metabolism-elevating dose, it does not rule out a difference in penetration of the hormone into cells.

SUMMARY

Manometric and colorimetric techniques have been used to evaluate the succinoxidase activities of a wide variety of tissues from euthyroid, thyroidectomized and thyroxine-injected rats.

A group of tissues including liver, heart, kidney, diaphragm, skeletal muscle and salivary gland exhibited succinoxidase changes paralleling the decrease in oxygen consumption observed in hypothyroidism and its increase in hyperthyroidism. Although pancreas belongs metabolically in this series, its succinoxidase values varied in the opposite directions.

Brain, spleen, ovary, uterus, testis, prostate, seminal vesicle, thymus and lymph nodes, all of which showed no oxygen consumption dependence upon thyroid status, displayed a more varied set of succinoxidase responses. In general, these did not correspond to thyroid hormone level.

The general conclusion from these findings is that the influence of thyroxine on tissue metabolism can be correlated in most tissues with the influence of the hormone on succinoxidase activity. There is no evidence as to how thyroxine can have such differing effects on the corresponding enzyme activity in various tissues.

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HISTOCHEMICAL DEMONSTRATION OF INSULIN IN THE ISLETS OF LANGERHANS[1]

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WITH the development of a new specific method for the histochemical demonstration of protein-bound sulfhydryl groups (Barrnett and Seligman, 1952), and disulfide groups (Barrnett and Seligman, 1954), the way was opened for the demonstration of individual proteins particularly rich in these reactive groups or of regions in tissue where such proteins were concentrated. The success or failure of such a demonstration would depend, in part, upon whether solution and extraction of the protein under the conditions of the method could be avoided.

In order to develop a method for demonstrating insulin in the islets of Langerhans, in various species and in various physiological states, we were encouraged by the facts that insulin, although deficient in sulfhydryl groups, is rich in disulfide by virtue of its 12% cystine content (du Vigneaud, Miller, and Rodden, 1939), that insulin was available in pure crystalline form for critical *in vitro* experiments to work out the details of the histochemical procedure, and that a good deal is known about the chemistry of insulin (Jensen, 1939; Sanger, 1949) and the changes in its concentration in the pancreas under different physiological conditions (Gomori, 1943; Haist, 1944; Lazarow, 1949; Houssay, 1950).

A description of the final histochemical method for the demonstration of insulin, including experiments designed to test its validity and to demonstrate its usefulness, form the subject of this paper.

MATERIALS AND METHODS

In vitro experiments

Solutions of insulin were made by dissolving 10 to 20 mg. of pure crystalline insulin[2] in 0.5 cc of .02 N hydrochloric acid as described by Miller and Andersson (1942). In

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[2] Crystalline insulin, Lot T2842, was kindly provided by Dr. Otto K. Behrens of the Lilly Research Laboratories, Indianapolis 6, Indiana.

a few instances acetic acid was used instead of mineral acid. The pH of the solutions was raised to 7.2 to 7.5 with veronal buffer and the volume to 10 cc with 0.85% sodium chloride. Various histological fixatives were added to the solution of insulin in excess of the amount required for precipitation. The precipitates were collected, either on filter paper or in centrifuge tubes, washed repeatedly in tap water and carried through each step used in the preparation of tissue sections and in the histochemical procedure for sulfhydryl and disulfide groups. The step of paraffin embedding was omitted. The precipitates were exposed to the various reagents for the same lengths of time required in the fixation, preparation and staining of the tissue sections.

Since glutathione, a sulfhydryl containing tripeptide, may be concerned with insulin synthesis (Lazarow, 1948), and could conceivably be present in significant concentration in tissue, *in vitro* experiments similar to those with insulin were conducted with pure glutathione.[3]

Histological and histochemical methods

Pieces of the tail of the pancreas were taken primarily from freshly killed normal rabbits and a few specimens of pancreas were also collected from normal albino rats, Swiss mice, dogs and humans at autopsy, as well as principal islets of the toad fish (*Opsanus tau*). These tissues were fixed for 24 hours in Romeis' fluid or a modification of this fixative and an occasional piece of pancreas was fixed in 80% ethyl alcohol containing 1% trichloracetic acid (80-TCA), neutral 10% formalin or Bouin's fluid. The tissues subjected to fixatives containing picric acid were washed in tap water overnight and all the pieces of pancreas were dehydrated and embedded in paraffin in the routine manner. Sections cut at either 5 or 10 μ were mounted and deparaffinized.

Ten μ sections from blocks fixed in 80-TCA or formalin were stained for sulfhydryls alone (Barrnett and Seligman, 1952) and for both sulfhydryls and reduced disulfides (Barrnett and Seligman, 1954) with the specific reagent, 2,2′-dihydroxy-6,6′-dinaphthyl disulfide (DDD).[3] Pancreases fixed in Romeis' fluid or its modification or Bouin's fluid were stained by the method for both sulfhydryls and reduced disulfides (Barrnett and Seligman, 1954). In addition, sections of rat pancreas were stained with the method for sulfhydryls and disulfides which utilized neotetrazolium as the reagent (Barrnett and Seligman, 1954).

Much of the information concerning the histology of islets under normal and abnormal circumstances has been obtained from examination of sections of pancreas stained with selective histological methods which reveal alpha and beta cells in the islet by staining the granules in those cells differentially. We have chosen one of these selective methods, the aldehyde fuchsin method of Gomori (1950), for comparison with the sulfhydryl and disulfide method. This staining method was found to give variable staining results according to the age of the aldehyde fuchsin solution, the length of time the section was exposed to an oxidizing agent, a reducing agent, and to the fuchsin reagent. After considerable trial with the variables of the aldehyde fuchsin method, we chose conditions similar to those described in the modification of the aldehyde fuchsin method by Wilson (1952). Although this modification is a satisfactory one, it was felt safer to stain all the sections, which were to be compared, with the same batch of aldehyde fuchsin. Trial 5 μ sections of normal pancreas were run for varying periods of oxidation, reduction and staining with aldehyde fuchsin that was 24 hours old and the conditions which gave maximum intensity of staining of beta cell granules were immediately repeated with the whole batch of experimental sections. Although the specific sulfhydryl and disulfide method (DDD) gives reproducible results, it was found convenient to make sections of all the blocks of pancreas reported here and stain them simultaneously.

[3] Schwarz Laboratories Inc., Mount Vernon, New York.

Specificity experiments

The reactions which were used to establish the specificity of the method for free sulfhydryls and for reduced disulfides have been described in *detail* earlier (Barrnett and Seligman, 1954, 1954a). In brief, the precipitates of insulin or the sections of pancreas did not develop color with the sulfhydryl method if: (1) the reduced precipitates or the reduced tissue sections were reacted with iodine, iodoacetate or N-ethyl maleimide before reaction with DDD, or if (2) the precipitates or sections already reacted with DDD were subjected to the reversing action of glutathione before reaction with tetrazotized diorthoanisidine.

Experimental alterations of insulin content

Adult male rabbits were used in six experiments designed to affect the insulin content of the pancreas. At the termination of the experiments, the rabbits were killed by an injection of air into the ear vein, and a piece of the tail of the pancreas was removed and fixed. In the alloxan treated and partially pancreatectomized animals, the presence of glycosuria was determined with Benedict's reagent at various intervals during the experiment and at autopsy a specimen of bladder urine was tested.

Rabbits were made diabetic by an intravenous injection of alloxan, 170–200 mg./kg. These animals were killed 4 hours, 8 hours, 1 ,2, 5, 8 and 34 days after the administration of alloxan. Other rabbits were given an intravenous infusion of 10% glucose in saline, equivalent to 5% of their body weight. The infusion by slow drip took 4 to 4.5 hours. A few rabbits were given the same injection of glucose intravenously in 10 minutes and were killed 20 minutes afterward.

Still other rabbits were deprived of all food but allowed water *ad libitum*. These were killed after a 7 or a 14-day fast. Other rabbits fed *ad libitum* were given intramuscular injections of protamine zinc insulin (Lilly) of 12 units per day for 14 days. In order to damage alpha cells, diethyldithiocarbamate (Kadota and Midorikawa, 1951) was also injected intravenously into rabbits. When a dose of 750 mg./kg. was used, almost all the rabbits died within 24 hours. The pancreases of these rabbits were not studied but those of ones given the same dosage and killed 6 to 10 hours after the injection were taken for study. A few rabbits who recovered from this treatment were killed 3 to 4 weeks after treatment.

A final group of rabbits was partially pancreatectomized. In each case, an attempt was made to remove more than an estimated 75% of the pancreas. Only five animals developed glycosuria postoperatively that lasted more than 10 days and only these were included in this study. These animals were killed 15, 16, 21, 28 and 35 days after operation and a piece of mesenteric pancreas was removed and fixed. Partial pancreatectomy is a particularly hazardous operation in the rabbit because of the intimate association of the tail of the pancreas with the blood vessels which supply the greater curvature of the stomach. It was only after considerable trial that a technique was developed for stripping these vessels without sacrificing them.[4]

The specimens of pancreas of the experimental rabbits were treated histochemically along with the pancreases of the normal control rabbits.

For the experiments on mice, the pancreases of obese hyperglycemic mice were taken for comparison with those of non-obese Swiss mice. In addition, the pancreases were also taken of Swiss mice and obese mice, both of which had received 15 mg. each of diethyldithiocarbamate 24 hours before they were killed.[5]

[4] Acknowledgement for aid in preparing partially pancreatectomized rabbits is due Mr. Thomas W. Barrnett.

[5] The authors wish to thank Drs. Jean Meyer and Stephen B. Andrus, Harvard School of Public Health, for providing the pancreases of obese mice and obese mice treated with diethyldithiocarbamate.

In vitro experiments on the fixation and staining of insulin

Although many protein precipitants will precipitate insulin from solution, most of these precipitates redissolved in the organic solvents used in the histological preparation of tissues or during the histochemical procedures. The precipitants which yielded permanently insoluble precipitates and which were most applicable to tissue work contained both picric acid and mercuric chloride. In the experiments reported here, the two precipitants or fixatives which were used were Romeis' fluid (45 cc. of saturated aqueous mercuric chloride, 40 cc. of saturated aqueous picric acid, 10 cc. formaldehyde, 5 cc. of glacial acidic acid) (Romeis, 1940) or a modification of Romeis' fluid in which 85 cc. of a stock aqueous solution was saturated with both mercuric chloride and picric acid.

Quantitative studies were not made on the precipitation of insulin, since determination of the nitrogen content of the supernatant could not be interpreted due to the large amount of nitrogen in the picric acid. The mercuric chloride interfered with the quantitative determination of sulfur in the supernatant. Accurate dry weight study of the supernatant was not feasible because of the high salt content of the fixative. However, some assurance of fairly complete precipitation of insulin was given by the observation that further precipitation was not produced by the addition of absolute acetone. The dry weight of the precipitated insulin in a series of experiments exceeded the weight of the crystalline insulin dissolved by as much as 23%, although the weight of the insulin precipitates in individual experiments varied widely. It was found that this variation could be caused by minor fluctuations in pH, temperature and concentration of precipitants. In addition, the neutralized supernatant of 5 cc. from which 20 mg. of insulin was precipitated showed no biological activity. The blood sugar of rats, $\frac{1}{2}$ hour, 3 hours and 5 hours after subcutaneous injection of the supernatant was not statistically different from the blood sugar of rats which were injected with the neutralized fixative to which no insulin had been added. However, the possibility exists that some insulin remained in solution after combination with the precipitating agents but was biologically inactive due to blockage of reactive groups.

The smallest amount of insulin that could be precipitated immediately by addition of the fixative at room temperature was determined by testing various dilutions of insulin solution. Visual recognition of the precipitate that formed limited the dilution to 4 μg. per cc.

The insulin precipitates formed by the addition of the fixative were insoluble in tap water, and in reagents used in dehydrating tissues prior to paraffin embedding, 80%, 95% and absolute ethyl alcohol and chloroform. Deparaffinization was performed on the precipitates as well, and it was found that they remained insoluble in xylene, a graded series of ethyl alcohols (absolute, 95% 80%, 70%) and water.

The precipitates were then subjected to the individual steps of the method for demonstrating both sulfhydryls and disulfides (Barrnett and Seligman, 1954) based on a specific method for sulfhydryls (Barrnett and Seligman, 1952). They were reduced by incubation for 2 hours at 37° C with freshly prepared thioglycolate (0.5 M), titrated to pH 8.5 with 0.1 N sodium hydroxide. There was no loss of insolubility of the precipitates during this incubation nor in the subsequent washes in acidified water (1% glacial acetic acid) or distilled water. The precipitates were then incubated in the buffer-alcohol solution of the specific sulfhydryl reagent (DDD). No loss in insolubility occurred. The precipitates remained insoluble during extraction of the excess of DDD and the reaction by product, 6-hydroxy-2-naphthyl thiol. The extraction was made with acidified water, for 5 minutes, a graded series of ethyl alcohols and absolute ether for 5 minutes, and again with the series of alcohols to water. Finally, the precipitate stained blue when tetrazotized diorthoanisidine (0.5 mg/cc) in Sorensen's phosphate buffer (pH 7.6) was added, demonstrating the reduced disulfides of insulin. The precipitates of insulin did

not stain with DDD and tetrazotized diorthoanisidine if the reduction with thioglycolate was omitted. Moreover, the insulin precipitate, which had been dehydrated and rehydrated did not stain with the aldehyde fuchsin method, demonstrating that this method is not specific for insulin, but rather for something else in the beta cell which accompanies insulin.

A saturated aqueous solution of picric acid, as in Bouin's fluid or a saturated aqueous solution of mercuric chloride, as in sublimate-formol, precipitated insulin from solution. However, the insulin-picrate precipitate was soluble in dilute ethyl alcohol (<93%) during the procedures of dehydration and rehydration. Some of the precipitate formed with sublimate-formol was lost during the procedure of reduction of the disulfides of the precipitate to sulfhydryls prior to staining them. Loss at either stage did not occur when a saturated aqueous solution of both picric acid and mercuric chloride was used as the precipitating agent. It was also noted that if thioglycolate was added directly to a solution of insulin (0.1%), a sulfhydryl containing precipitate developed slowly as insulin was reduced.

Although pure insulin contains no sulfhydryl groups, this was proven for the sample of insulin used, in the following manner: insulin, precipitated by absolute acetone and reacted with DDD in either absolute pyridine or a solution containing 95% acetone and 5% barbital buffer (pH 8.5) did not stain, whereas bovine serum albumin smeared on slides or a reduced precipitate of insulin, subjected to the same procedures, did stain.

Insulin crystals or insulin precipitated from solution by absolute acetone was completely soluble in a solution of 1% trichloracetic acid in 80% ethyl alcohol (80-TCA). No precipitate developed from a solution of insulin (0.1%), if 80-TCA or 10% neutral formalin was added. Insulin which had been precipitated by Romeis' fluid dissolved after 30 minutes at 36° C. in dilute sodium hydroxide solution (pH 11), containing 70% alcohol.

Glutathione (20 to 100 mg. dissolved in distilled water or isotonic saline) was not precipitated by Romeis' fluid or the more saturated modification. However, glutathione was precipitated when the fixative contained only mercuric chloride and picric acid, but the precipitate redissolved when either acetic acid or formalin, contained in the original fixatives, were added. When the fixative was added dropwise, a precipitate formed near the concentrated drop but redissolved as the reagent became diluted on stirring. A permanent precipitate of glutathione formed only when an excess of fixative was added and this turned gummy in consistency on standing. When this glutathione precipitate was subjected to the same steps of the procedure used in the *in vitro* staining of insulin, it dissolved completely; the major losses occurred during reduction or during extraction after reaction with DDD.

RESULTS

The distribution of sulfhydryl and disulfide groups in normal islets.

In sections of pancreas of rabbit, rat (Fig. 1) and mouse fixed in 80-TCA or formalin, the islets stained weakly for sulfhydryl groups in comparison to the acinar tissue. The staining of the islet cells was predominantly cytoplasmic and different cell types of the islet could not be distinguished on the basis of staining. Although the material which stained in the cytoplasm of these cells appeared granular under the oil immersion objective, this granular material was similar to that seen in the acinar cells of the pancreas and in the epithelial cells of other organs (Barrnett, 1953). Similarly fixed sections stained for both sulfhydryls and disulfides showed

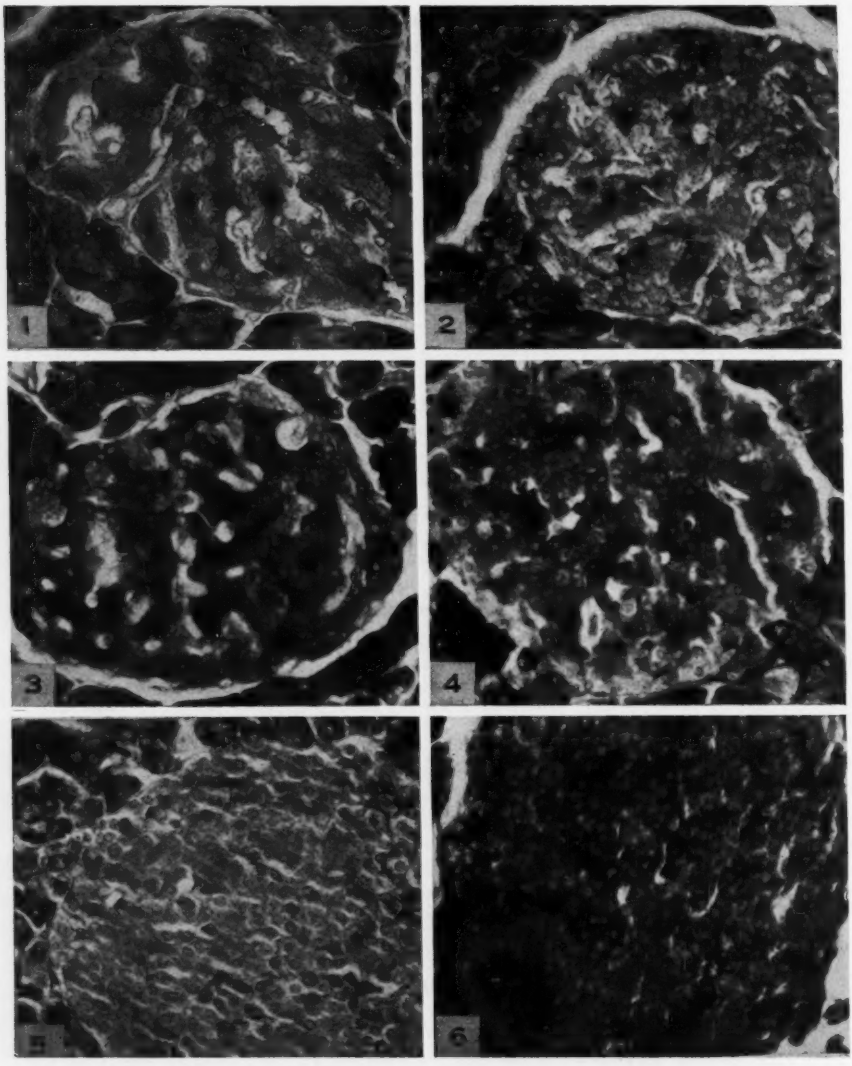

PLATE I

Photomicrographs (X440) demonstrate islets in 10μ sections of pancreas. All indicate the localization of both sulfhydryl and disulfide groups except Figure 1 which was stained only for sulfhydryls.

FIG. 1. Normal rat islet. Alcohol-TCA fixation. All the islet cells are weakly stained for sulfhydryls and the different cell types cannot be distinguished on the basis of staining. Acinar cells are most reactive in their basilar parts and are more intensely stained than islet cells.

FIG. 2. Normal rat islet. Alcohol-TCA fixation. There is virtually no increase in

a slight overall intensification of the reaction in islet cells, without any change in the distribution (Fig. 2). Islet cells did not stain with aldehyde fuchsin after these fixatives. Sections of pancreas fixed in Bouin's and stained by the method for both sulfhydryls and disulfides, were somewhat more intensely stained than 80-TCA or formalin fixed sections. However, there were still no significant differences between the staining of any of the cells. With the aldehyde fuchsin method, beta cells were revealed and differentiated from alpha cells by their purple (fuchsin) stained cytoplasmic granules. The alpha cell granules stained with light green S. F. (National Aniline).

Islets in sections of pancreas fixed in Romeis' fluid or its modification and stained for both sulfhydryls and disulfides showed a different distribution and intensity of reaction. In the normal rabbit, most of the islet cells were more intensely stained than either the acinar cells or the islet cells from sections of blocks fixed in 80-TCA, formalin or Bouin's fluid. In the cytoplasm of most of the cells, a material was retained by fixation in Romeis' fluid which reacted intensely. Three distinct types of distribution of the stained material were observed. These were: perinuclear staining, uniform staining of the entire cytoplasm or very concentrated staining in the part of the cytoplasm nearest a capillary (Figs. 7, 14, 18). Under the oil immersion objective, this reactive material was only suggestively granular and, therefore, different from the fuchsin staining of discrete granules. In most of the islets of the rabbit, almost all of the cells contained reactive material, although there was some variation as to the amount of it in the individual cells. In some islets, the cytoplasm of all the cells contained reactive material. However, in other islets, there were a few cells, usually located in the periphery, that did not contain this reactive material in their cytoplasm and stained weakly (Figs. 7, 14, 18). When sections of

staining in islet cells for both sulfhydryls and disulfides over that of sulfhydryls alone. Compare with Figures 1 and 3.

FIG. 3. Normal rat islet. Romeis' fixation. Contrary to Figures 1 and 2 in which the insulin was dissolved during fixation, this islet in which insulin has been retained, and stained, shows an intense reaction in the beta cells. The peripherally-placed, smaller alpha cells are less reactive. Contrary to normal rabbit (Figs. 7, 14, 18) acinar cells contain more sulfhydryl and disulfide groups than islet cells.

FIG. 4. Normal Swiss mouse islet. Sublimate-formal fixation. With a fixative that preserves insulin *in situ*, fewer sulfhydryl and disulfide groups are seen in the mouse beta cells than in the rat (Fig. 3) or the rabbit (Figs. 7, 14, 18). Peripherally placed alpha cells are poor in sulfhydryl and disulfide groups.

FIG. 5. Islet of obese-hyperglycemic mouse. Sublimate-formol fixation. In comparison with a normal mouse islet (Fig. 4), the obese mouse islet shows few sulfhydryl and disulfide groups.

FIG. 6. Islet of obese-hyperglycemic mouse treated with diethyldithiocarbamate. Sublimate-formol fixation. There is a pronounced increase in the sulfhydryl and disulfide groups of the beta cells as a result of this therapy.

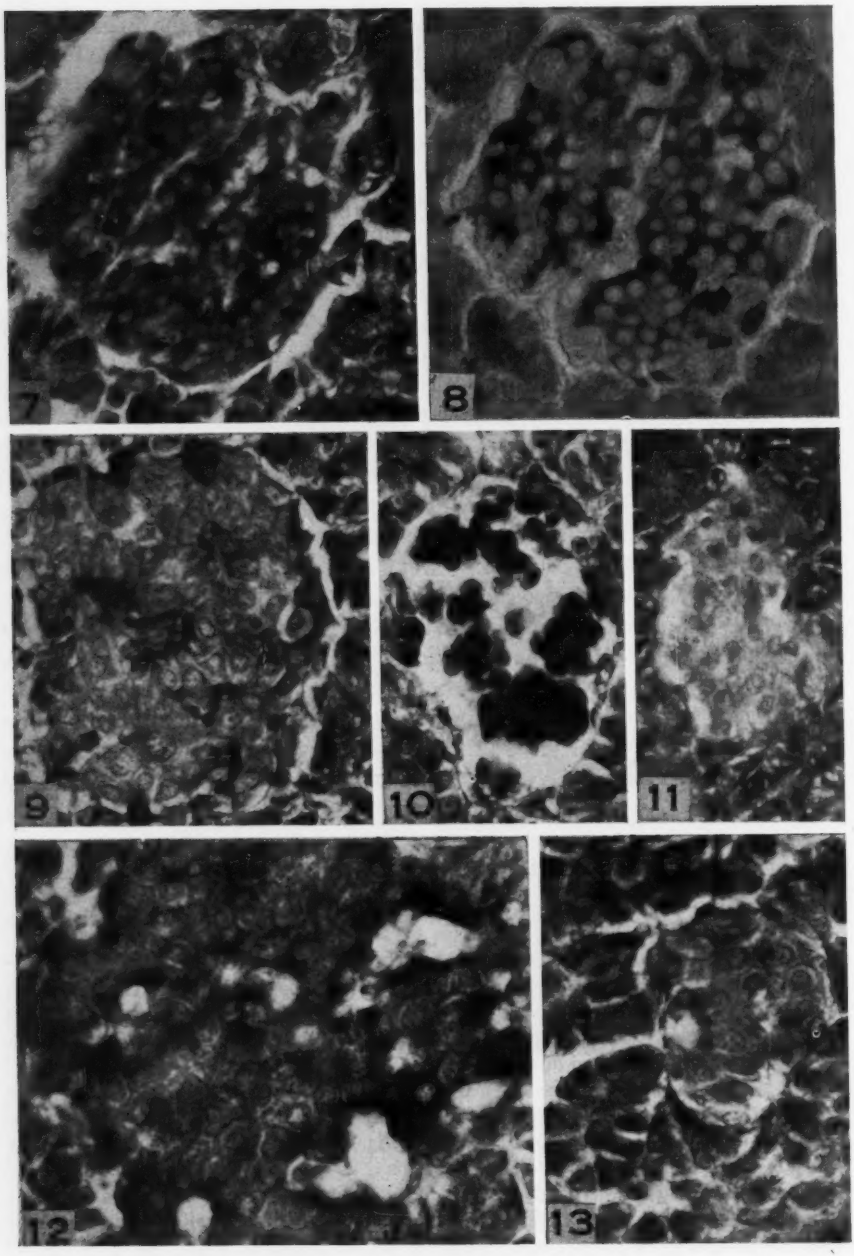

PLATE II

All photomicrographs (X440) demonstrate islets in sections of rabbit pancreases fixed

rabbit pancreas were treated with the method used to dissolve the insulin precipitates, as described in the *in vitro* experiments, all the cells of the islets showed a weak cytoplasmic reaction (Fig. 9) and appeared like the rabbit islets from blocks of pancreas fixed in 80-TCA and stained for sulfhydryl and disulfide groups (Fig. 2).

Similarly fixed sections stained with the aldehyde fuchsin method revealed the beta cells of the islets (Fig. 8). More intensely stained granules were present in the beta cells after this fixation in comparison to Bouin's fluid. The distribution of the stained granules in the cytoplasm was much like that of the intensely reactive sulfhydryl-disulfide material, but the intensity of staining with aldehyde-fuchsin was greater than the histochemical method. In the rabbit pancreas, it was noted that over 50% of the islets were composed of only beta cells in confirmation of the work of Jaffe (1951). In the remaining islets there were few alpha cells located predominantly in the periphery of the islet, where they occurred as single cells or small groups of a few cells. Consecutive sections of pancreas were stained with the two methods in an effort to deduce whether the cells which were intensely or weakly stained for sulfhydryl and disulfide corresponded to beta and alpha cells, respectively, with the selective staining method. This comparison was difficult because the sections stained for sulfhydryls and disulfides, were 10μ in thickness for the development of sufficient intensity of color, whereas the sections stained with aldehyde fuchsin were 5μ.

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in Romeis' fluid. All are stained for sulfhydryl and disulfide groups except Figure 8 which was stained with aldehyde fuchsin.

FIG. 7. Normal islet showing the distribution of intensely reactive sulfhydryl and disulfide-positive protein in the cytoplasm of most cells. At the extreme left of the islet there are a few, smaller cells that are weakly reactive.

FIG. 8. Normal islet showing beta cells stained with aldehyde fuchsin. Consecutive section to Figure 7 allowing the cells in both figures to be compared, and indicating that the beta cells are the ones rich in sulfhydryl and disulfide groups.

FIG. 9. Normal islet treated in dilute, alcoholic sodium hydroxide before staining for sulfhydryls and disulfides. The precipitated insulin has been dissolved and the staining of the islet cells appear much like those in which the insulin has been dissolved by the fixative (Fig. 2). Erythrocytes and acinar cells retain stainable protein.

FIG. 10. Islet 4 hours after the administration of alloxan. The islet has lost its normal architecture, is shrunken and the cells are pyknotic. The characteristic cytoplasmic reaction for sulfhydryls and disulfides in the normal beta cells (Fig. 7) has decreased. The acinar cells are less reactive than normal.

FIG. 11. Islet 2 days after the administration of alloxan. No sulfhydryl and disulfide positive material remains in any of the islet cells.

FIG. 12. Islet 34 days after the administration of alloxan. This animal which initially had severe glycosuria that abated in degree showed a partial return in sulfhydryl and disulfide-positive material in the islet beta cells which were larger than normal.

FIG. 13. Islet 10 hours after the administration of diethyldithiocarbamate. This treatment caused a decrease in the amount of sulfhydryl and disulfide-positive material.

In spite of the technical difficulties in matching the same cells in adjacent 5 and 10μ sections of an islet, it appeared that most of the beta cells which stained with aldehyde fuchsin (Fig. 8) also reacted strongly for sulfhydryls and disulfides (Fig. 7). In some instances, however, it appeared that an occasional beta cell reacted weakly in the histochemical procedure. The smaller alpha cells reacted weakly for sulfhydryl and disulfide groups. This conclusion was permissible because it was usually possible with the histochemical procedure to distinguish between the occasional lightly stained beta cells and the usual lightly stained alpha cells on the basis of size and position in the islet.

Islet cells in rat (Fig. 3) and mouse pancreases (Fig. 4) which were fixed in Romeis' fluid or its modification and stained for sulfhydryls and disulfides, were more intensely stained than after 80-TCA (Fig. 2) formalin or Bouin's solution. However, the intensity of the staining of these cells was less than that seen in the rabbit islet, and also less than that in acinar cells of the same animal. In the rat (Fig. 3) the cells which contained strongly reactive sulfhydryl and disulfide material in their cytoplasm occurred in compact cords that occupied all but the periphery of the islet. Yet all of the cells in the central cords did not react with uniform intensity, and occasional cells with lightly stained cytoplasm occurred among the darkly stained ones. In the periphery of the islet, smaller cells with lightly stained cytoplasm predominated (Fig. 3). In the mouse the islets were more compact than in the rat. The beta cells stained less intensely for sulfhydryls and disulfides than those of the rat and there were fewer weakly reactive, peripherally placed alpha cells (Fig. 4).

There was a good correspondence between the small, peripherally placed alpha cells and the larger centrally placed beta cells stained by the selective staining method, on the one hand, with the cells which were lightly and intensely reactive, respectively, with the histochemical method in both the rat and mouse. A few cells were exceptions, as noted for the rabbit.

A positive granular cytoplasmic reaction occurred in the rat beta cells, but not alpha cells, with a modification of the tetrazolium method (Barrnett and Seligman, 1954) in which a red-blue insoluble formazan was produced by reduction of neotetrazolium with reducing groups. Slnce it had already been found that alkali would dissolve insulin which had been precipitated by Romeis' fluid and since the original tetrazolium method utilized a solution of potassium cyanide and sodium hydroxide for the splitting of disulfides as well as the reaction of sulfhydryls with tetrazolium salts, the method was modified in the present experiments so that the incubating solution containing neotretrazolium and potassium cyanide (1–2%) was titrated to pH 8.5 with hydrochloric acid. Experiments to prove that the reduction of neotetrazolium was only produced by the reaction with free sulfhydryls and split disulfides, were not performed. How-

ever, the sites of staining with this method were the same as with the specific method (DDD).

Similar staining of large granules in the beta cells with both the selective histological method and the histochemical method was obtained on the principal islet of the toadfish (*Opsanus tau*). The beta cells of the islets of dog and man stained like those of the other mammals described. The intensity of the reaction of the beta cells for sulfhydryl and disulfide groups in the various species was in descending order; toadfish, rabbit, dog, rat, man and mouse.

Experimental Alterations of the Insulin Content of the Islets

Alloxan Administration. Four hours after an intravenous injection of alloxan, the islets lost their normal architecture. They appeared smaller and the cord-like arrangement of cells was completely disrupted. At this time, the beta cells were shrunken and showed a decrease in their cytoplasmic reaction for sulfhydryls and disulfides and an increase in the staining of these groups in the nucleus (Fig. 10). This type of nuclear reaction is characteristic of pyknotic cells. In the *eight hour* specimens, cell shrinkage and pyknosis were more pronounced and there was very little cytoplasmic reaction for sulfhydryls and disulfides. *One day* after alloxan, karyolysis and disintegration and disappearance of cells was prominent. In these early stages after the administration of alloxan, some of the specimens showed a decrease in sulfhydryl and disulfide groups in acinar cells (Fig. 10). Sections of the 2, 5 and 8 day specimens stained with the histochemical method showed that all the cells of the islet stained lightly for sulfhydryl and disulfide (Fig. 11). Complete absence of beta granules in these cells, stained with the selective method, indicated that the islets were composed of alpha cells and some degranulated beta cells. In the 34 day specimen numerous islets were present but most of these were smaller than normal but many of the islet cells were larger than normal. In the sections stained with the histochemical method, intensely positive sulfhydryl and disulfide material was present in the cytoplasm of some cells, especially the parts of the cells adjacent of blood vessels (Fig. 12). There was a good correspondence between the results at each of these stages obtained with the histochemical method and that obtained with aldehyde fuchsin, except in the first 24 hours after the administration of alloxan when large aggregates of material occurred in some islet cells and stained selectively but not histochemically.

Glucose administration. The islets of the rabbits which received glucose intravenously over a 4-hour period showed a decrease in beta granules with the selective stain and a decrease in sulfhydryl and disulfide-positive material with the histochemical method (Fig. 17). These results were more marked in the animals which received the glucose within 10 minutes.

Starvation and insulin administration. The starved and insulin injected rabbits all showed a decrease in granules and sulfhydryl and disulfide-

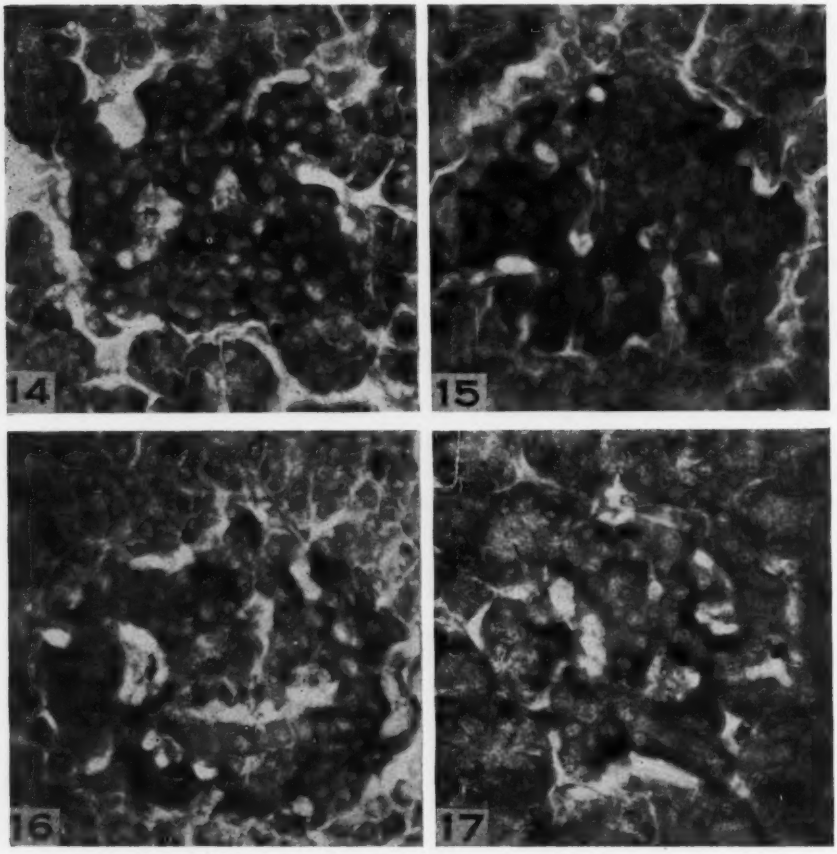

PLATE III

All photomicrographs (X440) demonstrate islets of rabbit pancreases fixed in Romeis' fluid. All are stained for sulfhydryl and disulfide groups.

FIG. 14. Normal islet. For comparison with Figures 15–17. Note that in the rabbit islet contrary to those in the rat (Fig. 3) and mouse (Fig. 4), the beta cells contain more sulfhydryl and disulfide-positive material than do the acinar cells.

FIG. 15. Islet of rabbit starved for 14 days. Less reactive material is present than in the normal.

FIG. 16. Islet of rabbit injected with 12 units of insulin daily for 14 days. This treatment caused a decrease in the sulfhydryl and disulfide-positive material in the islet beta cells.

FIG. 17. Islet of rabbit infused with 10% glucose solution for four hours. There is a reduction in the amount of reactive material in most of the islet cells.

positive material in beta cells. The sections from 14-day starved animals showed slightly less selectively and histochemically stained material (Fig. 15) than the sections from seven-day starved animals, and the sections from the insulin injected animals showed the least (Fig. 16). It was also

noticeable, especially in the selectively stained sections, that there were more alpha cells in the islets of the fourteen-day starved animals than either the seven-day or normal animals. Rabbits starved for 14 days and refed for 5 days had islet cells that stained the same as controls.

Diethyldithiocarbamate administration. The islets of the rabbits injected the same day with diethyldithiocarbamate showed a decrease in the histochemically reactive material (Fig. 13) and in the selectively stained granules of the beta cells. Although no change was noted in the alpha cells with the histochemical method, decreased granulation was noted with the selective staining method. The rabbits which recovered from the administration of diethyldithiocarbamate all received intravenous glucose therapy for the first two days. When they were killed, 3 to 4 weeks later, the beta cells showed either a normal or an increased staining for sulfhydryl and disulfide material.

Partial pancreatectomy. The rabbits which were killed 15 and 35 days after partial pancreatectomy showed a slight increase over the normal in beta cell granulation with the selective stain and a pronounced increase in sulfhydryl and disulfide-positive material histochemically in the islets (Figs. 19, 20). The beta cells of the 35-day specimen were enlarged while those of the 15-day specimen were of normal size. In sections of the other three specimens (16, 21, and 28 days postoperative), the beta cells of the islets contained fewer granules and less sulfhydryl and disulfide-positive material than normal (Figs. 21, 22, 23). None of the beta cells in any of the islets had pyknotic nuclei, and mitotic figures were present in the islets of the 28-day specimen (Fig. 22).

Obese mice. The islets in the pancreases of obese mice (Fig. 5) were more numerous and larger than in non-obese Swiss mice and also showed a significant reduction in beta granules and sulfhydryl and disulfide-positive material in comparison to the non-obese mice (Fig. 4). Twenty-four hours after the administration of 15 mg. of diethyldithiocarbamate, slight degranulation and decrease in sulfhydryl and disulfide reaction was seen in the islets of Swiss mice. In the islets of obese mice treated with diethyldithiocarbamate, on the other hand, there was a striking increase in beta cell granulation and in the amount of sulfhydryl and disulfide positive material (Fig. 6) in comparison to untreated obese mice.

No differences in the staining for sulfhydryls and disulfides could be observed when normal and experimental pancreases were fixed in 80-TCA or in Romeis' fluid followed by treatment with sodium hydroxide and alcohol before they were stained.

DISCUSSION

The present experiments show that more sulfhydryl and disulfide containing protein is present in the beta cells than in the alpha cells of the islets of Langerhans of the pancreas of six species of normal animals, pro-

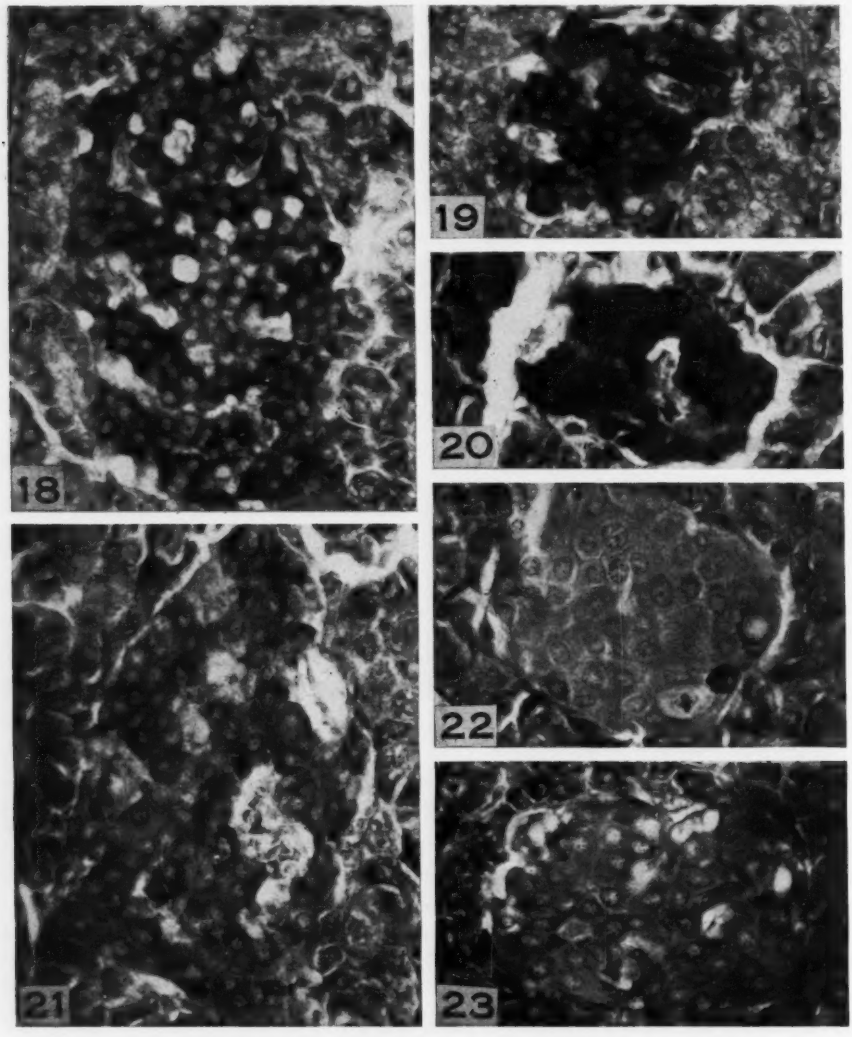

PLATE IV

All photomicrographs (X440) demonstrate islets of rabbit pancreases fixed in Romeis' fluid. All are stained for sulfhydryl and disulfide groups.

FIG. 18. Normal islet. For comparison with figures 19–23.

FIG. 19. Islet of pancreatic remnant 15 days after partial pancreatectomy, demonstrating a slight increase in the amount of sulfhydryl and disulfide groups in islet beta cells.

FIG. 20. Islet of pancreatic remnant 35 days after partial pancreatectomy, demonstrating a moderate increase in the amount of sulfhydryl and disulfide groups in islet beta cells which are larger than normal.

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vided the pancreases are fixed under conditions that render insulin insoluble. In addition, it was shown by *in vitro* experiments that the disulfide groups of insulin could be reduced to sulfhydryls which were stained with a specific histochemical method and during these precedures loss of insulin from re-solution was insignificant. Although some precipitated proteins such as keratins (Hardy, 1952) and neurohypophyseal protein hormones (Barrnett, 1954) can be rendered soluble by the prolonged action of reducing agents, such as thioglycolate, this was not the case with insulin precipitated by mercuric chloride and picric acid. Since insulin is precipitated from solution during reduction with thioglycolate (Miller and Andersson, 1942) this step only served to increase the insolubility of insulin, especially since it had been already precipitated by mercuric chloride and picric acid.

When the pancreases were fixed in solutions that did not precipitate insulin or precipitated it so that it was subsequently dissolved in organic solvents, fewer protein-bound sulfhydryl and disulfide groups were demonstrable in the cytoplasm of islet cells. Under these conditions, the cytoplasm of both alpha and beta cells were stained with equal intensity. Insulin precipitated by Romeis' fluid could be made soluble in dilute alcohol and sodium hydroxide, and when followed by staining for sulfhydryls and disulfides, the islets appeared the same as those in sections subjected to fixatives which did not precipitate insulin. In addition, it was shown in the *in vitro* experiments that the insulin appeared to be completely precipitated and the conditions used were effective on low concentrations of insulin.

Although our experiments indicated that insulin could be retained and stained in the beta cells of appropriately fixed pancreases, the possibility exists that after fixation in Romeis' fluid other sulfhydryl or disulfide-containing proteins besides insulin may be precipitated and stained. Furthermore, glutathione, which is present in beta cells in low concentration (Lazarow, 1946), may be depleted from the beta cells in the synthesis of insulin (Lazarow, 1948). It was important to be sure that glutathione was not being demonstrated under the conditions of the method. This was amply demonstrated in the *in vitro* experiments. If glutathione were combined with proteins through its amino or carboxyl group so as to leave its sulfhydryl free, it would be stained as protein sulfhydryl, not as glutathione.

FIG. 21. Islet of pancreatic remnant 16 days after partial pancreatectomy, demonstrating a moderate reduction in the amount of sulfhydryl and disulfide groups in islet beta cells.

FIG. 22. Islet of pancreatic remnant 28 days after partial pancreatectomy, demonstrating a pronounced reduction of sulfhydryl and disulfide groups in islet beta cells. Note the mitotic figure (metaphase) in an islet cell at the lower part of the photomicrograph.

FIG. 23. Islet of pancreatic remnant 21 days after partial pancreatectomy demonstrating a moderate reduction in the amount of sulfhydryl and disulfide groups in the islet beta cells.

If a sulfhydryl or disulfide containing protein with similar solubilities accompanies insulin, this stainable material in the beta cells would not be expected to vary in amount in the various physiological experiments exactly as insulin does. If the content of such a protein were unaffected by various physiological conditions, its presence would be a constant factor which could be differentiated from insulin by the experimental alteration of insulin content of the beta cells. No suggestion of such a substance was observed, although a sulfur-containing protein associated with insulin cannot be ruled out by our experiments.

The histochemical method for both sulfhydryls and disulfides utilizing DDD as reagent (Barrnett and Seligman, 1954), was used in these experiments in preferance to the method for disulfides alone (Barrnett and Seligman, 1954), even though it is less specific for insulin. The latter method requires the blocking of free sulfhydryls by an alkylating agent, such as iodoacetate and the splitting of naturally occurring disulfides by potassium cyanide. Since the principal requirement in choosing a fixative was that it precipitate insulin irreversibly for the conditions used, it would not be likely that one could find a fixative that didn't block or oxidize other sulfhydryls while precipitating insulin. This was the case for many protein-precipitating agents tested. The use of alkylating blocking agents to eliminate interference of sulfhydryl-containing protein under these conditions would have been fortuitous.

The mercuric chloride in our fixative presumably formed mercaptides with the free sulfhydryls in the tissue. However, these were made available for reaction with our reagent by the strong reducing agent, thioglycolate, which has been used by others to reduce the disulfides of insulin (Wintersteiner, 1933; Fraenkel-Conrat, 1943). For these reasons, we have referred to the groups in the tissues which were demonstrated as both sulfhydryls and disulfides even though there are no sulfhydryl groups in insulin. The demonstration of insulin is apparent only after comparison of sections in which insulin has been retained with sections in which insulin has not been retained and sections in which the content of insulin has been experimentally altered. The tetrazolium method for revealing protein-bound sulfhydryl and disulfide groups (Barrnett and Seligman, 1954) gave similar staining of beta cells in normal rat islets as did the specific method which utilized DDD as reagent.

The morphology and selective staining of the islets of the normal mammals with aldehyde fuchsin is in agreement with the descriptions given by Gomori (1939). The relative intensity of staining of the beta cells of the various species for sulfhydryls and disulfides as well as the selective stain of Gomori corresponded roughly to the relative insulin content of the pancreases given for these same mammals as determined by extraction and bioassay (Haist, 1944). Both the insulin content and the aldehyde fuchsin staining of beta cells was least in man and mouse and greatest in the

rabbit. Similarly stained material occurred in the beta cells of the principal islet of the toadfish.

The experiments designed to alter the content of insulin in the islet cells are an integral part of our hypothesis that insulin can be demonstrated histochemically. These experiments also provide a means of cross-checking the variations in the intensity of the histochemical reaction with the results of others who hypothesized a change in the insulin content on the basis of selective stains for beta granules or on the basis of insulin bioassay. With the exception of the alloxan treated rabbits, the change in the amount of stainable sulfhydryl and disulfide groups occurred only in beta cells of the islet. In the alloxan treated rabbits the staining of the extra-islet tissues of the pancreas was somewhat decreased. Alloxan reacts with at least sulfhydryl groups (Patterson, Lazarow and Levey, 1949).

The immediate decrease and final complete obliteration of the stainable sulfhydryl and disulfide groups of beta cells provides morphological evidence for the decrease in insulin content of the pancreas during the hypoglycemic state shortly after the administration of alloxan (Hughes, Ware and Young, 1944) and thereafter when diabetes is established (Haist, 1944; Lukens, 1948). In the rabbit killed 34 days after the administration of alloxan, in which beta cells with a moderate content of sulfhydryls and disulfides were found in the islets, less than an effective dose of alloxan was undoubtedly used. Similar partial effects of small doses were also reported by Houssey, Brignone and Mazocco (1946).

The interpretation of the results of the experiment, in which rabbits received glucose intravenously, is similar to that of Woerner (1938) and Dohan and Lukens (1947). Since hyperglycemia causes the liberation of insulin from beta cells, it should result in the observed decrease in staining for sulfhydryls and disulfides. Starvation for 7 or 14 days, which caused a decrease in the histochemical reaction in the beta cells, has been noted to decrease the insulin content of the pancreas (Best, Haist and Ridout, 1939; Haist, Ridout and Best, 1937). Insulin injections, which in these experiments decreased the insulin content of the beta cells, have also been shown to decrease the granules of beta cells (Haist and Best, 1941) as well as the content of insulin in the pancreas (Haist and Best, 1940).

The changes in the beta cells of rabbits given diethyldithiocarbamate are similar to those described by Kodota and Midorikawa (1951), and the results of the experiments on normal and obese mice, both untreated and diethyldithiocarbamate treated, are the same as those obtained by Mayer, Andrus and Silides (1953). The latter authors consider that the degranulation of the beta cells of the obese mouse is due to hypersecretion of insulin presumably in compensation for hypersecretion of the hyperglycemic factor. We observed that when the alpha cells are damaged by diethyldithiocarbamate, the granulation of the beta cells as well as the sulfhydryl and disulfide content increases markedly.

Two changes were observed in the histochemical staining of beta cells in the pancreatic remnant of partially pancreatectomized rabbits. The protein-bound sulfhydryl and disulfide groups either increased or decreased. The increase presumably was due to stimulation of the beta cells in substituting for the whole pancreas, whereas the decrease was due to excessive demand and exhaustion. These results are in agreement with the work of Homans (1914), Allen (1922) and Bell, Best and Haist (1942).

The results obtained with the histochemical method and the method which selectively stained beta granules in both normal animals and experimentally-treated animals were remarkably similar. In normal animals, the beta cells which were heavily granulated and stained with aldehyde-fuchsin, also showed an intensely positive cytoplasmic reaction for sulfhydryls and disulfides. The alpha cells showed neither reaction. When either of the two reactions increased or decreased in the beta cells of the experimentally treated animals, the other reaction increased or decreased in a like manner. This emphasizes the close relation between the granular content of beta cells (Gomori, 1943), the sulfhydryls and disulfide-containing protein, and the insulin content of the whole pancreas as shown by assay (Haist, 1944).

However, there were certain differences between the histochemical reaction and the selective staining with aldehyde-fuchsin that should be noted. The selective method stained obvious granules, whereas the material which was positive for sulfhydryl and disulfide groups was less granular. If, indeed, the same material was being demonstrated by both methods, the lack of granularity with the histochemical method could have been due to special treatment in some of the steps of the procedure; e.g. reduction with thioglycolate. However, there is no evidence that the same material is being demonstrated by the two methods. There is evidence to the contrary. If insulin is being stained in the beta cells by the histochemical method which is able to stain insulin *in vitro*, a different material, possibly an associated protein, must be stained with aldehyde-fuchsin since the dye does not stain insulin *in vitro*.

The present work indicates the profitable use of specific histochemical methods, supplemented by information gained from *in vitro* analysis and physiological experiments, to determine the topographical localization of certain specific hormones. Similar procedures are being used to study the islets of rats with various endocrine imbalances, as well as to demonstrate the localization of the hormones of the adenohypophysis (Ladman and Barrnett, 1954, 1954a; Barrnett, Ladman and McAllaster, 1955).

SUMMARY

The present studies demonstrate by *in vitro* experiments that insulin, precipitated from solution by Romeis' fluid, remains insoluble during the histological procedures necessary for the preparation of tissue sections and during the histochemical steps necessary for the staining of sulfhydryl and disulfide groups.

When the results of the *in vitro* experiments were applied to sections of the pancreas of six species of normal animals, it was found that the beta cells of the islets of Langerhans were strongly positive for sulfhydryl and disulfide groups whereas, the alpha cells were not. The intensity of histochemical staining of the islet beta cells of the normal animals corresponded to the insulin content as determined by bioassay by other investigators. If the pancreas was fixed in media which did not precipitate insulin irreversibly, under the conditions used, both alpha and beta cells stained weakly.

The specificity of the histochemical method for sulfhydryls and disulfide groups utilizing DDD as the reagent was proven for precipitated insulin *in vitro* as well as on sections of pancreas. A less specific method for sulfhydryls and disulfides utilizing tetrazolium salts, for which the specificity was not proven, gave similar staining results on sections of rat pancreas.

In addition, experiments designed to alter the insulin content of the pancreas were conducted so that the alterations were revealed by changes in the amount of reactive material with the histochemical method. These experiments on rabbits included the administration of alloxan, glucose, insulin, or diethyldithiocarbamate, starvation, and partial pancreatectomy. The experiments on mice included both obese, hyperglycemic and Swiss mice, both controls and treated with diethyldithiocarbamate.

In both normal and experimental animals when the histochemical staining of the sulfhydryl and disulfide groups was compared with that of aldehyde-fuchsin, the results were similar in that the same cells stained. In the experimental animals, the results of both stains were directly proportional. The two methods differed in some other respects, the most notable of which was that aldehyde-fuchsin did not stain insulin *in vitro*.

On the basis of *in vitro* and histochemical studies of the pancreas of normal and experimentally treated animals and comparison of these results with those of others who stained beta cells selectively or who bioassayed the pancreas for insulin content, it is concluded that the disulfide groups of insulin in islet beta cells have been demonstrated.

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STIMULATION OF THE RELEASE OF CORTICOTROPIN FROM THE ADENOHYPOPHYSIS BY A NEUROHYPOPHYSIAL FACTOR[1]

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WE HAVE previously observed (Saffran and Schally, 1955a) that neurohypophysial tissue plus arterenol (norepinephrine) strongly stimulate the release of corticotropin (ACTH) from anterior pituitary tissue *in vitro*. A corticotropin-releasing factor (CRF) has been found in posterior pituitary extracts and has been shown to be distinct from vasopressin and oxytocin.

METHOD

The detection of CRF activity is described in detail by Saffran and Schally (1955a). It consists of incubating rat anterior pituitary tissue with the test substance in bicarbonate-buffered, oxygenated medium at 38° for 1 hour in the presence of 0.0004 M DL-arterenol.HCl and 0.004 M ascorbate and then measuring the ACTH released into the medium, using the *in vitro* bioassay of Saffran and Schally (1955b). The results are expressed in milliunits (MU.) of ACTH released by 1 mg. of anterior pituitary tissue in 1 hour, accompanied by the 95% confidence limits of the ACTH assay. In most experiments the anterior pituitary tissue from each animal was distributed equally between two flasks, one of which served as a control, since the two halves of the gland behave almost identically under the same conditions (Saffran and Schally, 1955a).

In the doses used, the posterior pituitary preparations had no detectable ACTH activity when tested by the method of Saffran and Schally (1955b).

RESULTS

Crude posterior pituitary extracts

The corticotropin-releasing activity of the neurohypophysis does not require surviving tissue; the activity can be demonstrated in a water extract of rat neurohypophysis (Table 1). The extract was prepared by grinding the neurohypophyses of 6 rats, total tissue weight, 4.5 mg., in 75 μl. of water, using a few grains of sand and a glass rod to disintegrate the tissue. Thirty μl. of the extract were incubated for 1 hour with adenohypophysial tissue ± arterenol. With the water extract alone, 18.1 MU. of ACTH were released; arterenol increased the release by the extract to 27.4 MU. These figures are compared in Table 1 with the release by adenohypophysial

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TABLE 1. EFFECT OF THE NEUROHYPOPHYSIS AND ARTERENOL ON THE RELEASE OF ACTH

Additions	No. of tests	ACTH released, mU./mg. anterior pituitary	95% confidence limits, mU.
None	8	5.2	4.2– 6.2
Arterenol	3	7.2	4.7–10.8
Neurohypophysial tissue	4	9.0	6.4–14.1
Neurohypophysial tissue + arterenol	2	**25.3**	14.5–44.0
Water extract of neurohypophysis	1	**18.1**	12.0–27.5
Water extract of neurohypophysis + arterenol	1	**27.4**	13.4–55.8

The values in boldface type are significantly greater ($P \leq 0.05$) than the release of ACTH with arterenol alone.

tissue alone, with arterenol, and with arterenol plus fresh neurohypophysial tissue.

Acetone powders of beef and hog posterior pituitaries contain CRF (Table 2). The powders were extracted by heating with 0.08 M acetic acid, as described by Stehle (1933) for the extraction of vasopressin and oxytocin from the posterior pituitary. Small amounts of these extracts exhibited CRF activity, although, with both extracts, larger doses apparently inhibited the release of ACTH from anterior pituitary tissue *in vitro*.

Posterior pituitary hormones

Vasopressin and oxytocin, prepared from beef posterior pituitaries by Stehle and Fraser's (1935) procedure, had little CRF-activity by themselves (Table 3). However, Stehle's vasopressin (200 pressor units/mg.) plus arterenol stimulated ACTH-release; the oxytocin had no great activity even with arterenol (Table 3). After further purification (Benfey 1953) the vasopressin (400 pressor units/mg.) had lost most of its CRF activity.

Paper chromatography

The chromatographic systems used by Benfey (1953) to purify vasopres-

TABLE 2. CRF IN ACETONE POWDERS OF BEEF AND HOG POSTERIOR PITUITARY

Species	Dose γ acetone powder	Arterenol	ACTH released, mU./mg. anterior pituitary	95% confidence limits, mU.
Beef	12.5	present	**68**	41–113
	125	present	14	9– 23
Hog	50	present	**29**	14– 59
	1000	present	9	6– 14

The values in boldface type are significantly greater ($P \leq 0.05$) than the release of ACTH with arterenol alone.

TABLE 3. CRF IN POSTERIOR PITUITARY HORMONE PREPARATIONS

Preparation	Dose γ	Arterenol	ACTH released, mu./mg. anterior pituitary	95% confidence limits, mu.
Vasopressin, Stehle	50	absent	11.4	7.0–18.8
Oxytocin, Stehle	50	absent	13.8	8.4–23.1
Vasopressin, Stehle	50	present	**31.9**	18.5–55.0
Oxytocin, Stehle	50	present	10.5	4.8–23.0
Vasopressin, Benfey	22	absent	8.7	6.2–12.1
Vasopressin, Benfey	22	present	8.7	4.7–16.3

The value in boldface type is significantly greater ($P \leq 0.05$) than the release of ACTH with arterenol alone.

sin were applied to the separation of CRF activity from Stehle's vasopressin.

Several spots of 750γ each of Stehle's vasopressin were placed on a wide sheet of Whatman #1 filter paper and were developed with a mixture of butanol-acetic acid-water (4:1:5) by descending chromatography for 72 hours. Alternate lanes were sprayed with ninhydrin. The vasopressin area yielded the orange-yellow color of tyrosine while the other stained areas were the characteristic purple color of most amino compounds. The paper was cut as illustrated in Figure 1A and the sections were eluted with water and were tested for CRF activity. The area of greatest CRF activity was above that of vasopressin and clearly separate from it.

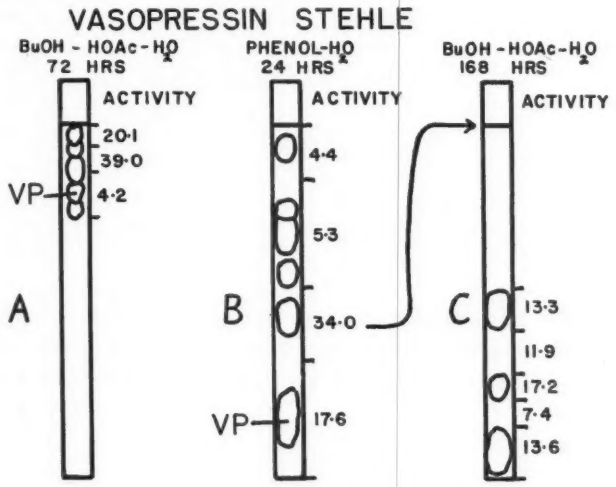

FIG. 1. Localization of CRF activity on paper chromatograms of vasopressin prepared according to Stehle and Fraser (1935). The spots were visualized by spraying with ninhydrin.

TABLE 4. RECOVERY OF MATERIAL FROM A CHROMATOGRAM OF STEHLE'S VASOPRESSIN IN THE PHENOL-WATER SYSTEM

Fraction	Yield mg.	Dose γ	ACTH released, mU./mg. anterior pituitary	95% confidence limits, mU.
I	5	100	4.4	0.6–34.0
II	50	100	5.3	2.5–11.2
III	15	100	**34.0**	18.3–63.7
IV	125	100	**17.6**	12.7–24.6
Total	195			

The values in boldface type are significantly greater ($P \leq 0.05$) than the release of ACTH with arterenol alone.

The bulk of the CRF activity could also be separated from the pressor area by chromatography in the phenol-water system. Two Whatman #3 wide sheets of filter paper were streaked at the starting line with 100 mg. each of Stehle's vasopressin and the chromatograms were developed with water-saturated phenol for 24 hours. Strips were cut from the sheet and were sprayed with ninhydrin. The paper was cut as in 1B and was eluted with water. The aqueous eluates were evaporated to a few ml. under reduced pressure, 10 ml. of methanol were added, and the material in the eluate was precipitated by the addition of ethyl acetate to a volume of about 100 ml. The precipitates were collected by centrifugation and dried *in vacuo*. The yield of the eluted solids was practically quantitative (Table 4). Only fraction III exhibited marked CRF activity (Figure 1B); the vasopressin-containing fraction IV had some activity. Rechromatography of fraction III in the butanol-acetic acid-water system for 92 hours yielded

TABLE 5. ENHANCEMENT OF THE CRF ACTIVITY OF A VASOPRESSIN-FREE MATERIAL BY ARTERNOL

Arterenol	ACTH released, mU./mg. anterior pituitary	95% confidence limits, mU.
Absent	**19.4**	11.3–33.3
Present	**30.5**	18.7–49.6

The values in boldface type are significantly greater ($P \leq 0.05$) than the release of ACTH with arterenol alone.

3 ninhydrin-staining areas (Figure 1C); the middle area exhibited moderate CRF activity, while tests on the other areas were not conclusive.

The activity of a vasopressin-free fraction (Figure 1A) is also enhanced by arterenol (Table 5).

DISCUSSION

McCann and Brobeck (1954) were the first to observe that large doses of Pitressin[2] could reproduce the normal pituitary-adrenocortical response

[2] Parke-Davis preparation of vasopressin.

(eosinopenia and adrenal ascorbic acid depletion) in animals rendered unresponsive to other stimuli by discrete hypothalamic lesions. They therefore concluded that vasopressin acts in the release of ACTH from the adenohypophysis and that relatively large doses are required to overcome the rapid destruction of the hormone. However, they point out that much smaller doses were required to reverse the diabetes insipidus that developed in these animals. This would imply that the ACTH-releasing site of action of vasopression is much less sensitive than the antidiuretic site of action. On the basis of our experiments the explanation of McCann and Brobeck's findings probably lies in the contamination of Pitressin by the CRF of the posterior pituitary. If this explanation is correct, it implies that CRF is active *in vivo* as well as in our *in vitro* system. Rothballer and Skoryna (personal communication) have administered our vasopressin-free fraction III (Table 4) to a dog rendered unresponsive to other stimuli by destruction of part of the median eminence (pituitary stalk section) and, when given along with epinephrine, they have obtained a significant eosinopenia.

The data in this paper support our previous suggestion (Saffran and Schally, 1955) that the neurohypophysis is an important link in the pituitary-adrenal response to stress and that arterenol (or epinephrine) acts synergistically with the CRF in releasing ACTH. The problem of the control of CRF discharge remains to be attacked.

Previous studies (summed up by Harris and Fortier, 1954) have pointed to the localization of the control of ACTH-release in the hypothalamus. We (Saffran and Schally, 1955) and Guillemin (1955) have found CRF activity in the hypothalamus, but the activity of the neurohypophysis is much greater. It is likely that, as is postulated for oxytocin and vasopressin (Scharrer and Scharrer, 1954), the CRF is elaborated within neurosecretory cells in the hypothalamus and is stored in the neurohypophysis to be released on demand. Alternatively, the endings of the hypothalamicohypophyseal tracts may release arterenol and/or epinephrine after a stressful stimulus and the sympathetic amine, in turn, causes the secretion of CRF from the neurohypophysis and acts with it in the discharge of ACTH.

The chemical nature of the CRF is unknown. Two pieces of evidence suggest that it is a peptide. Firstly, CRF contaminates vasopressin, which has been concentrated by methods known to separate peptides. Secondly, the activity is closely related to ninhydrin-staining areas on paper chromatograms in two solvent systems.

SUMMARY

Posterior pituitary extracts contain a corticotropin-releasing factor (CRF) that stimulates the release of ACTH from rat anterior pituitary tissue *in vitro*.

Vasopressin, prepared by the method of Stehle and Fraser, contains CRF as an impurity.

CRF can be separated from Stehle's vasopressin by paper chromatography and has been shown to be distinct from vasopressin and oxytocin.

The activity of CRF is enhanced by arterenol (noradrenaline).

The evidence suggests a role of the neurohypophysis in the pituitary-adrenal response to stress.

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ON THE THERAPEUTIC VALUE OF ACTH AND CORTISONE IN EXPERIMENTAL BURNS[1,2]

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MEDICATION with adrenocorticotrophic hormone and cortisone has been recommended for a great variety of pathological conditions, including severe burns, where even the exclusive use of such drugs has been advocated (Whitelaw, 1951). However, a critical survey of the clinical reports on which these claims are based reveals that in most of the cases hitherto described either the severity of the thermal injury was not convincingly established by the usual criteria of hypotension, hemoconcentration and reduction of urinary output, or the concomitant application of other treatments as, for instance, infusion of large volumes of plasma confounded the issue. Justified criticism on these grounds has been voiced by Tamerin (1951) and others. As there is no reliable standard by which the severity of burn in man may be quantitatively assessed and, moreover, as no patient can be left without a treatment of recognized efficacy to serve as a control, it is obvious that only the laboratory experiment can decide the merits of a new medication. Indeed, there have been some experimental investigations on the use of hormones in the treatment of burn shock, but most of these deal with less homogeneous hormonal preparations than are now available, so that antagonistic activities of the multiple constituents of such substances (Knowlton *et al.*, 1949) might have been responsible for the failures reported, or the design of the experiments was unnatural, i.e., therapy was commenced long before the supposed accident occurred, or other forms of post-traumatic treatment were applied simultaneously.

EXPERIMENTAL

The investigation was carried out on white mice of about 20 mg. of bodyweight with a method similar to that described for rats by Neal *et al.* (1952). In each experiment 100 animals were used, 25 for each type of treatment. The back of the ether-anesthetized mouse was immersed in hot water for a few seconds, carefully avoiding contact between water and the animal's anal region and head. The latter is necessary lest the animals should rapidly die from overheating of the nervous centers, the former to avert subse-

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TABLE 1. RESULTS OF EACH TYPE OF TREATMENT IN TERMS OF ACCUMULATED DEATHS

No. of experiment	Dose of hormone (mg./kg.)	Repetition of treatment after 24 hrs.	Sex of animals	Observation time (days)	Temperature of water (° C.)	Duration of exposure (sec.)	Day of experiment	Treatment			
								Saline control	ACTH	ACTH + Cortisone*	Cortisone
I	25.0	no	♂	6	92–93	2.5	1	14	14	9	14
							2	23	21	16	20
							3	23	23	17	21
							5	24	23	17	24
							6	24	23	18	24
II	25.0	yes	♀	9	95–97	2.5	1	1	6	4	6
							2	5	11	10	12
							3	6	12	**15**[1]	**14**[2]
							5	6	12	**16**[3]	**14**[2]
							7	6	12	**17**[4]	**14**[2]
III	10.0	yes	♀	7	94–95	3.0	1	5	8	6	13[5]
							2	13	16	16	18
							3	14	20	19	19
							4	15	20	20	19
IV	3.0	no	♀	3	95–96	3.0	1	22	23	24	19
							2	25	25	25	24
							3	25	25	25	25
V	3.0	yes	♂	8	95–96	2.5	1	13	14	14	**21**[6]
							2	22	20	23	24
							3	22	21	24	24
							6/7	22	23	24	24
							8	22	24	24	24
VI	0.5	yes	♀	9	96–97	2.5	1	2	3	7	4
							2	4	6	11	7
							3/4	7	7	13	9
							8	7	7	14	9
Total of experiments no. I, II, III, IV, V, VI							1	57	68	64	**77**[7]
Total of experiments no. I, II, III, V, VI							1	35	45	40	**58**[8]
Total of experiments no. II, III, V, VI							2	44	53	**30**[9]	**61**[10]

* Mixture of equal parts of both hormones, giving the total dose indicated in second column.
Encased figures indicate that result is significantly different from that of saline control, P being [1]0.022, [2]0.043, [3]0.010, [4]0.005, [5]0.039, [6]0.034, [7]0.020, [8]0.003, [9]0.023, [10]0.016.

quent bacterial infections and difficulties in defecation and urination. Immediately after scalding, the animals were injected subcutaneously at a site not exposed to the hot water and in a volume of 10.0 ml./kg. with saline solutions of adrenocorticotrophic hormone,[3] saline dilutions of a commercial cortisone suspension,[4] a mixture of both hormones, or physiological saline. The harmlessness of the highest dose of hormones had been previously ascertained (Schöttler, 1954). In most of the cases, this treatment was repeated after 24 hours. Further treatment was deemed unnecessary because it was evident after 48 hours that the hormones did not exert a beneficial effect on the injured animals. Special care was taken to get unbiassed results by keeping the operators who did the anesthesia and the scalding unaware of whether they were handling an animal destined to treatment or to serve as a control. The mice were kept under observation till death or complete systemic recovery. Food and water were always at the animals' disposal.

More frequent hormone applications at shorter intervals, were not considered necessary since Dougherty and White (1944) have demonstrated the lasting effect of a single subcutaneous injection of an aqueous ACTH solution in the mouse and Scott and Engel (1953) that of a saline suspension of cortisone acetate in the rat for a 24-hour period. The reason for testing a mixture of both hormones was the observation of Sayers and Sayers (1947) that an increased blood level of cortical hormones suppresses the secretion of the adrenotrophic hormone by the pituitary gland.

[3] Generously supplied by The Armour Laboratories, Chicago, Ill., through the courtesy of Dr. R. J. Meyer.

[4] Cortone (Merck & Co., Inc., Rahway, N. J.)

RESULTS

The outcome of the experiments is recorded in Table 1. The statistical evaluation of these data by means of the χ^2-test against the null hypothesis, considering when necessary Yates' correction for continuity, reveals that there was no significant difference in the mortality rates of the ACTH treated and the untreated mice. On the other hand, treatment with cortisone proved to be deleterious in a number of cases. This becomes expecially evident if the results of all experiments are combined. Then, the hazard inflicted on the animals by cortisone is statistically significant after a single treatment irregardless of whether Experiment IV, where the trauma was so severe that it killed all of the animals, is included in the analysis or not. After repetition of the medication, the detrimental effect of cortisone becomes statistically manifest also in the ACTH+cortisone treated animals, which had only received half as much cortisone as had been injected in the mice treated with this hormone alone. These results remained unchanged on prolongation of the observation time, so that at the end of the investigation P for the total of all experiments was 0.041 for cortisone and 0.020 for the cortisone+ACTH treated animals. If Experiment IV is excluded from statistical consideration the figures for P are 0.033 and 0.011 respectively.

DISSCUSION

An objection might be raised against the experimental technique employed in this investigation, namely that the time of the animals' exposure to the thermal injury was too short to be applied with meticulous precision. However, the only way to prolong the duration of exposure would have been either by diminishing the temperature of the water bath, or by decreasing the area of the animal which was submitted to the action of heat. Both of these possibilities were discarded for the following reasons. It was the object of this study to simulate as closely as possible the conditions under which life endangering burn accidents occur in the human being. This will generally be the short exposure of an appreciable part of the body surface to intense heat, such as that of steam or flames. Therefore, the use of the former of the above-mentioned alternatives would increase the discrepancy between the natural pathologic occurrence and the experimental procedure. On the other hand, the application of considerably higher degrees of heat, such as produced by overheated steam or the flame of a micro-burner, to a small area of a mouse's trunk is not feasible in so small an animal, where vital organs are situated within a few millimeters from the surface of the skin. Burning of a limb, however, might fail to provoke fatal trauma, analogous to the observations of Eijkman and Hoogenhuyze (1906), who reported death in bats after relatively mild thermal injury of their wings but survival after more severe scalding. Clearly the trauma to which the animals were submitted in this study was markedly more uniform than that of any group of patients available to clinical investigation.

A justification for the clinical trial of ACTH and cortisone in burn shock is supplied by the well-known concept of the influence of stress upon this hormonal system and by the vast experimental evidence that the application of these substances restores the lowered resistance of artificially hormone depleted animals to normal. However, this phenomenon does not necessarily imply that the resistance of normal individuals can be indefinitely raised by increasing their natural hormone supply. It is, further, an acknowledged fact that ACTH or adrenal cortical deficiencies as well as burns increase the permeability of the capillaries, but this coincidence does not mean that the mechanism involved is the same in both of these conditions. On the contrary, it has been convincingly explained in an ample discussion of this subject by Moon (1942) that the leakage of plasma from the circulation in the acute stage of burn shock is not due to hormonal insufficiency. Furthermore, the demands of hormone exerted by stress differ qualitatively with the type of stress as obvious from the experiments of Scherr (1952), who showed that cortisone protected mice from cold stress but lowered their normal resistance against heat stress. Under these considerations, the failure of the hormones in the treatment of burn shock, as evidenced by the results of the present investigation, can hardly be regarded as a disappointment, because the reasoning which led to their clinical use was unsound. Subjective improvement of patients might have been simulated by the euphoric state ACTH may produce according to Sprague *et al.* (1950). For the sake of completeness it must be mentioned that the findings reported in this paper agree with the results of the one similar experimental study (Neal *et al.*, 1952), where the daily administration of 5 mg. of cortisone in scalded rats did not reduce the latters' mortality in comparison with the untreated control animals.

The ineffectiveness of ACTH and cortisone when employed as the sole treatment in burn shock does not exclude the possibility that these hormones may be useful for maintaining normal capillary permeability once the deficient circulation has been restored by the infusion of a sufficient volume of liquid of adequate oncotic pressure as some authors believe, for instance Crassweller *et al.* (1950). The problem of the pathology of burns is not exclusively one of how to normalize the altered permeability of the capillaries, because this is not continuously increased along with the severity of the thermal injury (Schwiegk and Schöttler, 1947). They found that beyond a certain degree of heat intensity extravasation of fluid from the circulation stops entirely. At this point, the damage to the capillaries becomes probably irreversible and thus refractory to any form of therapy.

SUMMARY

Mice were scalded in hot water and afterwards treated with ACTH, cortisone or a mixture of both hormones in doses ranging from 0.5 to 25.0 mg./kg. In a total of 600 animals including controls, no beneficial

effect of hormone therapy was observed. Under the conditions of the experiment, cortisone proved to be contraindicated for the treatment of burns because it significantly enhanced mortality.

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RÔLE OF THE ADRENAL GLAND IN THE RESPONSE OF PLASMA POTASSIUM OF THE RAT TO MODERATE AND SEVERE HYPOXIA[1,2]

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KLINE (1952) has shown that, in the cat, moderate hypoxia produces a decrease in the plasma potassium concentration, whereas severe hypoxia produces an increase. He believes that these electrolyte shifts are influenced by secretions from the adrenal gland. More recently it has been demonstrated (Sussman *et al.*, 1953) that moderate hypoxia produces a decrease in plasma potassium concentration in the rat. The present investigation was undertaken in an attempt to determine the rôle of the adrenal in the response of plasma potassium of the rat to moderate and also to severe hypoxia.

METHODS

Male rats of the Wistar strain weighing betwen 200 and 300 gm. were used. They were fed Purina Chow and water *ad libitum*.

Hypoxia was produced by acute decompression whereby rats were taken to simulated altitudes of 25,000, 28,000, 30,000 or 35,000 feet within 2 to 3 minutes. After 30 minutes they were returned to ground level as quickly as possible and a blood sample was obtained. Some studies of severe hypoxia were conducted upon animals decompressed to simulated altitude of 40,000 feet until respiration ceased and then immediately returned to ground level.

Decompression was produced by partially evacuating a modified autoclave by means of three vacuum pumps. The desired simulated altitude was measured with a mercury manometer and regulated by a valve connecting the interior of the chamber with room air. The return to room atmospheric pressure was achieved within 15 to 30 seconds by opening the valve. Adequate ventilation of the chamber throughout decompression was maintained by the continual operation of all three pumps. It ranged from approximately 10 liters of air per minute at 40,000 feet to 25 liters at 25,000 feet.

Blood was obtained by decapitation, only the initial flow of blood being collected (2 to 2.5 cc.) in order to avoid contamination of the sample by surrounding tissue fluids

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Heparin was used as an anticoagulant, and all blood samples were immediately centrifuged in a clinical centrifuge at about 2800 R.P.M. The plasma was separated, and the concentration of potassium was determined by means of a flame photometer, using the internal standard method.

Bilateral adrenalectomies were performed in one-stage operations through a dorsal midline approach. The animals were anesthetized with 4 mg. of Nembutal per 100 gm. of body weight intraperitoneally. On completion of the operation, 0.5 cc. (150,000 units) of Depo-Penicillin was injected intramuscularly. Two to three weeks were allowed for recovery, during which time the animals were maintained on 1% NaCl.

Adrenaline (Parke, Davis and Company Adrenalin Chloride, 1:1000) was administered intraperitoneally, usually in dosages of 0.02 mg. per 100 gm. of body weight. An equivalent volume of physiological saline (0.02 cc. of 0.85% NaCl per 100 gm. of body weight) was given by the same route to control animals.

RESULTS

Effect of Moderate Hypoxia on the Intact Rat

Intact rats were divided into a control group and four experimental groups. The latter were decompressed to a simulated altitude of 25,000

TABLE 1. CHANGES IN THE MEAN PLASMA POTASSIUM CONCENTRATION OF RATS AFTER DECOMPRESSION TO VARIOUS ALTITUDES

	Intact		Adrenalectomized		Adrenalectomized adrenaline-injected	
	No. of animals	Plasma K m.eq./L.	No. of animals	Plasma K m.eq./L.	No. of animals	Plasma K m.eq./L.
Ground level	18	6.35 ± 0.10*	7	6.37 ± 0.12	8	5.31 ± 0.15
25,000 ft. for 30 mins.	11	5.83 ± 0.11				
28,000 ft. for 30 mins.	13	5.46 ± 0.16				
30,000 ft. for 30 mins.	12	5.34 ± 0.18	13	6.25 ± 0.18	6	5.11 ± 0.12
35,000 ft. for 30 mins.	5	4.95 ± 0.17				
40,000 ft. until respiratory collapse	7	9.59 ± 0.56	2	11.5		

* Standard error of mean.

28,000, 30,000, or 35,000 feet for 30 minutes. The control group was also placed in the chamber but was kept at room pressure for 30 minutes with the motors running, in order to eliminate any possible effect that might result from the stress of the noise and vibration of the decompression chamber. There was no sign of any physiological distress upon removing any of the animals from the chamber, except for the hyperpnea characteristic of the experimental groups.

The results are shown in Table 1. At 25,000 feet the mean plasma potassium concentration decreased from a control value of 6.35 m.eq./L. to 5.83 m.eq./L., or 8.2%. This fall was found to be significant to the 0.3% level, as determined by Fisher's analysis for a small sample. At 28,000

and 30,000 feet the decrease amounted to 14.0 and 15.9%, respectively, and reached a maximum of 22.0% at 35,000 feet. We may assume these changes are also significant, since they are much greater than that produced by exposure to 25,000 feet. Except for one case at 25,000 feet and another at 30,000 feet, all values for each of the experimental groups fell below the mean of the control group. Since exposure to 35,000 feet proved fatal to some animals, it was decided not to carry out experiments of this type at higher altitudes.

Effect of Severe Hypoxia on the Intact Rat

Several of the animals subjected to 35,000 feet for 30 minutes in the previous study showed signs of severe oxygen lack. Since previous studies by Kline (1952) on the cat have shown that decompression to the point of respiratory collapse leads to a rise in the plasma potassium concentration instead of a fall, it was decided to determine whether the rat behaved in a similar fashion. Consequently, 7 animals were taken to a simulated altitude of 40,000 feet until the onset of gasping, which usually occurred within 8 to 13 minutes. They were then immediately returned to room pressure and blood samples were taken. As shown in Table 1, the mean plasma potassium concentration increased to 9.59 m.eq./L., a rise of 51% when compared with the control value of 6.35 m.eq./L.

Effect of Moderate Hypoxia on the Chronically Adrenalectomized Rat

In order to determine whether the adrenal glands are involved in the marked fall in the plasma potassium concentration observed in intact rats when exposed to conditions of moderate hypoxia, the preceding studies were repeated on chronically adrenalectomized animals. The results are shown in Table 1. As indicated, the mean potassium levels of the intact and adrenalectomized groups are approximately the same (6.35 m.eq./L. and 6.37 m.eq./L., respectively). In addition the individual values in both series were found to be in the same range.

Exposure of the chronically adrenalectomized group to a simulated altitude of 30,000 feet for 30 minutes failed to reproduce the decrease in the plasma potassium concentration observed previously in the intact rats under identical conditions. Animals so treated and decapitated immediately on return to room pressure had a mean plasma potassium concentration of 6.25 m.eq./L., only 0.12 m.eq./L. below that of the corresponding controls (Table 1). This small difference was computed to be insignificant. Therefore, it seems likely that the adrenals are implicated in the fall in plasma potassium seen in the intact rat during moderate hypoxia.

In addition 2 animals were subjected to conditions of severe hypoxia by being exposed to a simulated altitude of 40,000 feet until respiration became labored almost to the point of respiratory collapse (Table 1). Their

plasma potassium concentrations were found to be 13.96 and 9.04 m.eq./L., respectively, values characteristic of the level observed in intact rats under identical conditions. This would indicate that the adrenals are not implicated in the marked increase in plasma potassium characteristic of severe hypoxia.

Effect of Adrenaline on the Chronically Adrenalectomized Rat

Chronically adrenalectomized rats were injected intraperitoneally with adrenaline in a dose of 0.02 mg./100 gm. of body weight. As shown in Table 1, after 30 minutes the mean plasma potassium concentration was reduced to 5.31 m.eq./L., a fall of 16.6% when compared with the control level of 6.37 m.eq./L. This decrease is of the same magnitude as that observed in the intact animal subjected to moderate hypoxia (Table 1).

Effect of Adrenaline on the Chronically Adrenalectomized Rat Exposed to 30,000 Feet

Since adrenaline produces approximately the same degree of fall in the mean plasma potassium concentration of the chronically adrenalectomized rat as did exposure to moderate hypoxia in the intact animal, a study was made of the effect of adrenaline on the plasma potassium concentration of the adrenalectomized animal subjected to moderate hypoxia. Six chronically adrenalectomized animals were injected intraperitoneally with 0.02 mg. of adrenaline per 100 gm. of body weight and then exposed to a simulated altitude of 30,000 feet for 30 minutes. At the end of this time, as shown in Table 1, the potassium level averaged 5.11 m.eq./L., a decrease of 19.8% below the control level of 6.37 m.eq./L. This decrease does not differ significantly from that seen when adrenaline is injected without decompression.

DISCUSSION

In the rat (Sussman *et al.*, 1953), as in the cat (Kline, 1952) and the dog (Ferguson and Smith, 1953), the oxygen lack resulting from decompression to simulated altitudes of 25,000 to 35,000 feet for 30 minutes or from inspiration of mixtures of 8 to 12% oxygen (McQuarrie *et al.*, 1940a) reduces the plasma potassium concentration significantly. From the results reported in the present study it is apparent that in the rat, as in the cat (Kline, 1952), the adrenal gland is involved in this response since it fails to occur after adrenalectomy. In adrenalectomized dogs, however, a fall in plasma potassium is still seen after decompression (Ferguson and Smith, 1955) and after breathing 5% oxygen (Ziegler, 1940; McQuarrie *et al.*, 1940b).

Although proof is not available, the weight of evidence favors the view that, in the rat, medullary rather than cortical activity is concerned. It is known that oxygen lack causes the release of adrenaline from the adrenal

glands (Cannon and Hoskins, 1911; Kellaway, 1918, 1919; Houssay and Molinelli, 1926; Bülbring *et al.*, 1948; Emerson and Van Liere, 1953). Furthermore, it has been shown that the injection of adrenaline, under certain conditions, reduces the plasma potassium in several animals and in man (D'Silva, 1934; Keys, 1938; Rogoff *et al.*, 1950; Dury, 1951; Dury and Johnston, 1951; Dury *et al.*, 1952). That this effect of injected adrenaline is not dependent upon the presence of the adrenal cortex is indicated by the fact that it can be reproduced in adrenalectomized animals (Fulton *et al.*, 1930; Rogoff *et al.*, 1950; Dury, 1951). This point has been confirmed in the present investigation, where it was found that intraperitoneal injection of adrenaline in doses of 0.02 mg. per 100 gm. of body weight into adrenalectomized rats produced a fall in the plasma potassium concentration of approximately the same magnitude as that produced in the intact animal by moderate hypoxia.

Kline (1952) attributes the drop of plasma potassium in cats after decompression to the release of adrenal cortical rather than medullary hormones, since he still notes a 14% drop in plasma potassium in splanchinicectomized cats after moderate decompression. Zwemer and Newton (1928) have shown, however, that oxygen lack will release adrenaline from the denervated gland.

While adrenaline has been shown to reduce plasma potassium concentration, immediate increases after adrenaline injections have also been reported (D'Silva, 1934, 1936; Keys, 1938; Rogoff *et al.*, 1950; Kline, 1952). Rogoff and his co-workers (1950) believe that when injected adrenaline causes an immediate rise in plasma potassium it is because the level of circulating adrenaline exceeds that produced by the average physiological rate of adrenaline secretion from the adrenals. If sufficient time elapses to allow the injected adrenaline to fall below a critical level, then the elevated potassium falls. Kline (1952) is among those reporting an early rise in potassium after adrenaline injection in the cat. In view of Rogoff's findings (1950) it is likely that if Kline had waited longer than 5 minutes he would have found the plasma potassium to have decreased, as it does in the rat 30 minutes after injection.

In two chronically adrenalectomized rats decompression to the point of respiratory collapse (40,000 feet) elevated plasma potassium markedly (13.96 and 9.05 m.eq./L., respectively). The same effect has been observed in chronically adrenalectomized dogs (Ferguson and Smith, 1955). It has also been reported in adrenalectomized dogs after clamping the trachea (Mullin, 1938) and in adrenalectomized cats after opening the chest wall (Cattell and Civin, 1938). Thus it would appear that in the rat and the dog the adrenals are not involved in the rise of plasma potassium seen at respiratory collapse. Kline (1952) reports, however, that in the acutely adrenalectomized and anesthetized (Nembutal) cat there is only an 11% rise, as compared to a 54% rise in non-anesthetized intact cats. The reduced response might be attributed to the anesthesia instead of the lack

of the adrenal medullae if it were not for the fact that unanesthetized splanchnicectomized cats also show only an 11% rise in plasma potassium at respiratory collapse (Kline, 1952). The difference between the behavior of the plasma potassium at respiratory collapse in the cat as compared to the rat and the dog remains to be explained.

SUMMARY

In the unanesthetized, intact rat decompressed to simulated altitudes of 25,000, 28,000, 30,000 and 35,000 feet for 30 minutes there is a significant reduction in plasma potassium concentration. In the unanesthetized, chronically adrenalectomized rat there is no change in the plasma potassium concentration after decompression to a simulated altitude of 30,000 feet for 30 minutes. Injection of adrenaline (0.02 mg./100 gm.) into the adrenalectomized rat reduces the plasma potassium concentration by approximately the same amount as does moderate decompression in the intact rat.

When either intact or adrenalectomized rats are subjected to a simulated altitude of 40,000 feet to the point of respiratory collapse, there is a significant increase in the plasma potassium concentration.

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INTERACTION BETWEEN HYDROCORTISONE-4-C^{14} OR PROGESTERONE-4-C^{14} AND SERUM ALBUMIN AS DEMONSTRATED BY ULTRACENTRIFUGATION AND ELECTROPHORESIS

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SOLUBILITY studies in aqueous systems have demonstrated considerable interaction between various steroid hormones and serum albumin. Human plasma dissolved about 2.5 times as much hydrocortisone as water; a five-fold increase of the water-solubility was observed in a 25% human serum albumin solution (Macek *et al.*, 1952). Approximately 20 or 30 times the amount of progesterone, soluble in water, could be dissolved in 6% albumin or in serum, respectively (Bischoff *et al.*, 1954). The interactions with serum albumin and possibly other serum proteins result in maximal binding capacities of serum for these steroid hormones corresponding to about 1,000 to 10,000 times their physiological concentrations.

The present studies tend to clarify the status of interaction between hydrocortisone or progesterone and serum albumin at concentrations close to physiological. Such knowledge is considered essential for an understanding of the transport mechanism of the steroid hormones which in turn is believed to be related to their mode of action.

It has been shown recently by paper-electrophoretic and paper-chromatographic methods that only a small percentage of cortisol and almost the total amount of progesterone present in serum albumin-steroid mixtures is bound and thus transported by the protein (Westphal *et al.*, 1955). Some limitations of the procedures employing paper as a supporting medium (Westphal *et al.*, 1954) were avoided in the present studies by the use of ultracentrifugation and electrophoresis in "free solution."

EXPERIMENTAL

Materials and Chemical Methods. The properties of the hydrocortisone-4-C^{14},[1] the purification of the progesterone-4-C^{14} preparation[2] employed, and the methods of dis-

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[2] Obtained from Radioactive Products, Inc., Detroit, Michigan, on allocation from the Isotope Division, U. S. Atomic Energy Commission.

solving the steroids in the aqueous systems have been described (Westphal *et al.*, 1955). The steroid concentrations ranged from 0.6 to 5.0 μg. per ml. The same crystalline human serum albumin preparation[3] was used as in previous studies (Westphal *et al.*, 1955). The albumin concentrations in the experimental fractions were determined either by a micro-Kjeldahl procedure (Westphal *et al.*, 1952) or by the biuret reaction (Gornall *et al.*, 1949). A 10% solution of sulfosalicylic acid was employed to ascertain the absence of protein in the fractions subsequently referred to as free of albumin. The recoveries of albumin and of the steroid hormones are given in the tables.

Ultracentrifugation. A Model E Spinco Ultracentrifuge was used. Approximately 6.5 ml. of the solutions were pipetted into the plastic tubes ("Lusteroid") of the preparative rotor "J" and centrifuged for 5 hrs. at 56,100 RPM (average centrifugal force g = 197,000). The refrigeration of the machine caused the temperature to drop from approximately 26° C. at the start to approximately 20° C. at the end of the runs. No braking was applied after the centrifugation was completed.

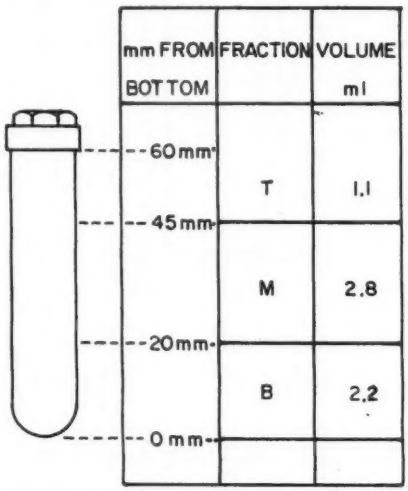

FIG. 1. Fractionation scheme in ultracentrifuge experiments.

Immediately after the rotor had come to a halt the plastic tubes were taken out of the rotor and their contents were sampled without removal of the caps. Three fractions were obtained as indicated in Figure 1. The top (T) and middle (M) fractions were recovered by piercing the tube with syringe-mounted No. 23 needles at the 45 mm. and 20 mm. levels, respectively. An air hole was pierced close to the 60 mm. level for pressure equilibration. In all experiments with albumin the high viscosity of the bottom fraction (B) required the use of a No. 20 needle. Figure 1 also shows the average volumes obtained in the three fractions.

Electrophoresis. The C^{14}-steroids were dissolved in a solution of 4.0% human serum albumin which had been dialyzed at 3° C. against Michaelis buffer of pH 8.6, $\mu = 0.1$. One volume of the steroid-albumin solution was then diluted with 2 volumes of the buffer. The experiments were done in a standard-size electrophoresis apparatus[4] using the long

[3] The author wishes to express his appreciation to Dr. F. F. Johnson, Cutter Laboratories, Berkeley, California, for supplying this material (Batch No. 262–89).

[4] Frank Pearson Associates, New York 12, N. Y.

analytical cell at 200 volts and 24 ma. for a period of 4 hrs. The content of the cell was sampled in 7 fractions as indicated in Figure 2. No. 23 and 20 needles were used for the top and lower fractions, respectively. Care was taken to avoid any disturbance of the cell content during sampling.

Determination of C^{14}-Steroids. The concentrations of the radioactive steroids in the various fractions were determined by direct plating and measuring the activity in a gas-flow counter, according to procedures outlined previously (Westphal *et al.*, 1955). A small correction for self absorption was applied to a few planchets that contained more than 2 mg. of albumin, using a correction curve which will be described elsewhere. The

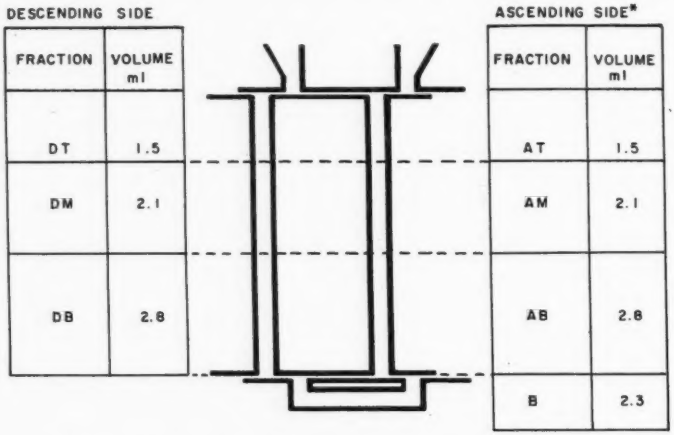

FIG. 2. Fractionation scheme in electrophoresis experiments.

* For convenience, the bottom fraction (B) is included on this side of the tabulation.

progesterone-4-C^{14} adsorbed to the "Lusteroid" tubes was recovered by extracting the dissected parts of the tubes 4 times with alcohol at 65° C.

RESULTS AND DISCUSSION

The paper-electrophoretic and paper-chromatographic procedures recently employed in a study on the interactions between serum albumin and steroid hormones (Westphal *et al.*, 1955) have several limitations. On the one hand, the small volumes of the solutions applied to the paper call for a comparatively high concentration of the C^{14}-steroids if the detection method is to be sensitive. On the other hand, the uncertainty about adsorption of the steroids to the paper prevents an estimation of the albumin-free portion of the hormones. Both of these disadvantages were avoided in the present investigation of the systems in "free solution."

In the following tables examples are given of the findings obtained in several ultracentrifuge experiments. Table 1A shows the interaction between hydrocortisone-4-C^{14} and albumin as observed in a mixture of the two components. In the top fraction (T) 34% of the cortisol originally present in this fraction was found after ultracentrifugation. Since no

TABLE 1. INTERACTION BETWEEN HYDROCORTISONE-4-C^{14} AND ALBUMIN AS DEMONSTRATED BY ULTRACENTRIFUGATION

Fraction[1]	Albumin			Hydrocortisone-4-C^{14}			
	Before Ultracentrifugation	After Ultracentrifugation		Before Ultracentrifugation	After Ultracentrifugation		
	mg.	mg.	% of original[2]	μg.	μg.	% of original[2]	μg. extracted from wall
A. 1.00 μg. Hydrocortisone-4-C^{14} per ml. 4% albumin in 0.9% NaCl (pH 6.6)							
T	48.0	0	0	1.20	0.41	34.0	—
M	112.0	27.4	24.5	2.80	1.31	46.7	—
B	84.0	221.2	263.3	2.10	4.41	210.0	—
Total	244.0	248.6		6.10	6.13		
% of Original		101.9			100.4		
B. 1.00 μg. Hydrocortisone-4-C^{14} per ml. 0.9% NaCl							
T				1.20	0.93	77.0	0.01
M				2.90	2.33	80.0	0.01
B				2.30	2.32	100.0	0.02
Total				6.40	5.58		0.04
% of Original					87.2		0.6

[1] Definition of fractions given in Figure 1.
[2] Refers to amount present in fraction before ultracentrifugation.

albumin was left in this fraction, 34% of the hydrocortisone obviously was present free of the protein. In contrast, the same experiment with progesterone-4-C^{14} (Table 2A) revealed that only 1.6% of this steroid was free in solution. Ultracentrifugation of hydrocortisone-4-C^{14} (Table 1B) and progesterone-4-C^{14} (Table 2B) in albumin-free solution failed to demonstrate any marked sedimentation of the steroids under the conditions employed. The different distribution of the two steroids (Tables 1A and 2A) is therefore caused by their different interaction with albumin.

A complication arose in the studies with progesterone-4-C^{14}. Only 26% of the total radioactivity could be recovered in the 3 fractions when the steroid was ultracentrifuged in albumin-free saline (Table 2B). It was found that a large part of the progesterone-4-C^{14} employed was firmly adsorbed to the "Lusteroid" tubes during the ultracentrifugation and could be extracted with alcohol. The "Lusteroid" tubes consist of cellulose nitrate which is known for its great affinity to ketones. The wall-adsorption of progesterone was much less in the presence of albumin (Table 2A). The explanation is believed to be competition between the cellulose nitrate and albumin for the diketo-steroid. The binding of progesterone to the protein reduced the wall-adsorption to 10% compared to almost 50% in albumin-free solution. The adsorption of progesterone was found to be independent of the ultracentrifugation. Practically no adsorption to the "Lusteroid"

TABLE 2. INTERACTION BETWEEN PROGESTERONE-4-C^{14} AND ALBUMIN AS DEMONSTRATED BY ULTRACENTRIFUGATION.

Fraction[1]	Albumin			Progesterone-4-C^{14}			
	Before Ultracentrifugation	After Ultracentrifugation		Before Ultracentrifugation	After Ultracentrifugation		
	mg.	mg.	% of original[2]	μg.	μg. in solution	% of original[2]	μg. extracted from wall
A. 0.67 μg. Progesterone-4-C^{14} per ml. 4% albumin in 0.9% NaCl (pH 6.6)							
T	40.0	0	0	0.67	0.01	1.6	0.08
M	100.0	3.5	3.5	1.68	0.09	5.1	0.17
B	84.0	219.5	261.2	1.41	2.96	211.0	0.14
Total	224.0	223.0		3.75	3.06		0.39
% of Original		99.6			81.6		10.4
B. 1.00 μg. Progesterone-4-C^{14} per ml. 0.9% NaCl							
T				1.00	0.22	21.8	0.65
M				3.10	0.82	26.5	1.17
B				2.00	0.57	28.6	1.19
Total				6.10	1.61		3.01
% of Original					26.4		49.3

[1] Definition of fractions given in Figure 1.
[2] Refers to amount present in fraction before ultracentrifugation.

tubes was observed with hydrocortisone-4-C^{14}.

An attempt was made to obtain a complete picture of the interaction between albumin and the two steroids on the basis of the ultracentrifugation studies (Table 3). The observation that 34% of the hydrocortisone was free in solution (Table 3A) justifies the assumption that 66% was bound to albumin. This assumption was tested by calculating the amount of hydrocortisone transported by that portion of albumin which sedimented into Fraction B. The result was 67% (Table 3A, Fraction "BU"), indicating the essential validity of the postulated ratio of free to bound hydrocortisone under the conditions employed. A similar calculation for the interaction between progesterone and albumin (Table 3B) was impaired by the complicating factor of wall-adsorption mentioned above. No accurate values, therefore, could be expected in this case.

The ultracentrifuge studies are believed to give reliable results for the free steroids. However, apart from the difficulty due to the adsorption of progesterone to the "Lusteroid" tubes, the calculation of the albumin-bound hormones is considered less accurate. Since the albumin molecules moved into an albumin solution under the force of the gravitational field, the amount of steroids transported by the sedimenting protein could be determined by difference only. This limitation was overcome by the use of electrophoresis in free solution.

TABLE 3. INTERACTION BETWEEN ALBUMIN AND HYDROCORTISONE-4-C^{14} OR PROGESTERONE-4-C^{14}, CONCLUDED FROM ULTRACENTRIFUGE EXPERIMENTS
Other details given in Tables 1 and 2

Fraction[1]	Steroid present as				
	Total	Free[2]		Bound	
	μg. per ml.	μg. per ml.	% of original total	μg. per 40 mg. albumin	% of original total
A. 1.00 μg. Hydrocortisone-4-C^{14} per ml. 4% albumin in 0.9% NaCl					
Original	1.000	0.340	34	0.660	66
T	0.340	0.340	34	0	0
M	0.467	(0.340)	(34)	0.520	52
B	2.100	(0.340)	(34)	0.670	67
BU[3]	1.100	0	0	0.674	67
B. 0.67 μg. Progesterone-4-C^{14} per ml. 4% albumin in 0.9% NaCl					
Original	0.670	0.010	1.6	0.660	98.4
T	0.010	0.010	1.6	0	0
M	0.034	(0.010)	(1.6)	0.674	102
B	1.410	(0.010)	(1.6)	0.536[4]	80[4]

[1] Definition of fractions given in Figure 1.
[2] Figures in parentheses connote the assumption that the values for the free steroids in Fractions M and B are equal to those in Fraction T.
[3] BU refers to that part of the albumin and hydrocortisone-4-C^{14} that was transported into Fraction B by ultracentrifugation (BU = B-(1.00 μg. hydrocortisone-4-C^{14} + 40 mg. albumin per ml.)).
[4] Value is low due to wall adsorption.

Tables 4 and 5 show the results of electrophoretic migration of albumin-steroid mixtures in buffer solution. Obviously, any steroid remaining on the

TABLE 4. INTERACTION BETWEEN HYDROCORTISONE-4-C^{14} AND ALBUMIN AS DEMONSTRATED BY ELECTROPHORESIS
1.84 μg. Hydrocortisone-4-C^{14} per ml. 1.2% albumin

Fraction[1]	Albumin			Hydrocortisone-4-C^{14}		
	Before Electrophoresis	After Electrophoresis		Before Electrophoresis	After Electrophoresis	
	mg.	mg.	% of original[2]	μg.	μg.	% of original[2]
DT	16.9	0	0	2.58	0.46	17.7
DM	35.0	0.1	0.26	5.34	1.25	23.3
DB	24.1	14.2	58.9	3.68	2.25	61.1
B	28.9	28.9	100.0	4.42	4.42	100.0
AB	0	21.6	∞	0	2.75	∞
AM	0	30.0	∞	0	2.52	∞
AT	0	7.2	∞	0	0.10	∞
Total	104.9	102.0		16.01	13.74	
% of Original		97.3			86.0	

[1] Definition of fractions given in Figure 2.
[2] Refers to amount present in fraction before electrophoresis.

TABLE 5. INTERACTION BETWEEN PROGESTERONE-4-C^{14} AND ALBUMIN AS DEMONSTRATED BY ELECTROPHORESIS

1.28 μg. Progesterone-4-C^{14} per ml. 1.3% albumin

Fraction[1]	Albumin			Progesterone-4-C^{14}		
	Before Electrophoresis	After Electrophoresis		Before Electrophoresis	After Electrophoresis	
	mg.	mg.	% of original[2]	μg.	μg.	% of original[2]
DT	19.5	0	0	1.92	0.01	0.8
DM	26.0	0	0	2.55	0.02	1.0
DB	37.7	22.2	58.9	3.70	2.22	60.0
B	28.6	27.5	96.2	2.81	2.88	102.4
AB	0	32.1	∞	0	3.01	∞
AM	0	25.2	∞	0	2.56	∞
AT	0	2.9	∞	0	0.27	∞
Total	111.8	109.9		10.98	10.98	
% of Original		98.4			100.0	

[1] Definition of fractions given in Figure 2.
[2] Refers to amount present in fraction before electrophoresis.

descending side (Fig. 2) in those fractions from which albumin had moved out, must have been free in the original solution. In the ascending limb the albumin was transferred into the protein-free buffer. Any steroid found on this side of the cell after the electrophoresis must have been transported there in an albumin-bound form. In Table 6 a compilation is made which demonstrates the degree of interaction between albumin and the two steroids. In the albumin-free fraction (DT) about 18% of the original hydrocortisone was found present in free form. After correction for the extremely small amount of albumin in Fraction DM (see Table 4), about 23% free hydrocortisone was observed in this fraction. On the ascending

TABLE 6. INTERACTION BETWEEN ALBUMIN AND HYDROCORTISONE-4-C^{14} OR PROGESTERONE-4-C^{14}, CONCLUDED FROM ELECTROPHORESIS EXPERIMENTS

Other details given in Tables 4 and 5

Fraction[1]	Hydrocortisone-4-C^{14}				Progesterone-4-C^{14}			
	μg. per 10 mg. albumin	Moles albumin per mole hydrocortisone	Free, in % of original	Bound, in % of original hydrocortisone-albumin ratio	μg. per 10 mg. albumin	Moles albumin per mole progesterone	Free, in % of original	Bound, in % of original progesterone-albumin ratio
Original	1,526	34	—	—	0.982	46	—	—
DT	∞	0	17.7	0	∞	0	0.8	0
DM	138.4	0.4	23.1[2]	—	∞	0	1.0	0
DB	1.585	33	—	—	1.001	45	—	—
B	1.526	34	—	—	1.046	44	—	—
AB	1.273	41	0	83.4	0.938	49	0	95.5
AM	0.840	63	0	55.1	1.017	45	0	103.5
AT	0.132	398	0	8.7	0.944	48	0	96.1
Ascending Side, Average	0.912	58	0	59.8	0.971	47	0	98.9

[1] Definition of fractions given in Figure 2.
[2] Corrected for the small amount of albumin-bound hydrocortisone on the basis of the original hydrocortisone-albumin ratio.

side, the binding values differed between the various fractions and ranged from 9 to 83% (Table 6). This indicates a very weak attachment of hydrocortisone to albumin; the advancing front of the albumin contains much less steroid than the following portions. Considering the whole of the ascending limb a transport value of about 60% was determined. This figure is in satisfactory agreement with the value of 66% observed in the ultracentrifuge studies.

In contrast only about 1% of free progesterone was found to be present in the albumin-free fractions DT and DM (Table 6). On the ascending side an average binding of 99% of the original was observed. Since the estimations in the descending and ascending limbs of the cell are independent of each other, this result also constitutes a check for the reliability of the procedure. It is also in agreement with the findings by the ultracentrifugal, paper-electrophoretic and paper-chromatographic methods.

It was of interest to correlate the binding of hydrocortisone and progesterone to the number of albumin molecules involved. It can be concluded from the available solubility studies that the maximum binding capacity of albumin for hydrocortisone (Macek *et al.*, 1952) and progesterone (Bischoff *et al.*, 1954) is approximately one mole steroid per mole albumin. As pointed out earlier, this ratio will never be reached under any physiological or pathological conditions short of hormone injections. The secreting glands apparently are not able to increase the hydrocortisone (Eik-Nes *et al.*, 1954a; Hudson and Lombardo, 1955) or progesterone concentration (Zander, 1954) more than to a level which is still several orders of magnitude below the maximum binding capacity of serum or albumin for these steroid hormones. Consequently, a great number of albumin molecules seem to participate in the attachment of a single hydrocortisone or progesterone molecule.

The average number of albumin molecules responsible for the binding of the two steroid hormones have been calculated and are presented in Table 6. The figures obtained show a wide variation in the case of hydrocortisone which is considered another indication of a very weak interaction. In contrast, the firm binding between albumin and progesterone is demonstrated by a close constancy in the number of albumin molecules per steroid molecule. At present it is difficult to understand the mechanism of an interaction in which so many, or one of so many, protein molecules seem to be involved in a binding of a single steroid molecule.

The present results indicate that the maximum binding capacities of serum albumin for hydrocortisone and progesterone were not utilized. A considerable percentage of hydrocortisone and a small but definite proportion of progesterone were present in free form even at concentrations which were several orders of magnitude lower than those employed in solubility studies. The observation of almost complete binding of progesterone to serum albumin is not considered contradictory to the conclusion of Hooker

and Forbes (1949) that about 90% of the plasma progesterone is present in free form, since these authors used a different definition of "binding," i.e., the failure to extract progesterone with organic solvents.

The ratio of free to albumin-bound progesterone was found to be about 1 to 100. Close agreement was observed between the ultracentrifugal and electrophoretic methods in establishing this value, which also is in general agreement with the results of the paper-electrophoretic and paper-chromatographic studies (Westphal *et al.*, 1955). Apparently a strong attachment between progesterone and albumin is responsible for the constancy of the binding ratio found under the various experimental conditions. In the case of hydrocortisone, however, such constancy was not demonstrable. The weak interaction between albumin and the highly polar steroid seems to be the cause for the considerable variation in the ratio of free to bound hydrocortisone. The binding values obtained for this steroid should be considered as essentially of a qualitative nature. This is illustrated by the different percentage binding observed in the three anodic fractions as shown in Table 6.

The difference in the degree of albumin binding observed for the two steroids of different "polarity" may explain the limited renal clearance for progesterone compared to that of more hydrophilic steroids like hydrocortisone or steroid glucuronides. This suggestion is based on studies employing the steroids at physiological concentrations. Similar conclusions have been drawn by Eik-Nes, Schellman, Lumry and Samuels (1954b) from studies on steroid binding to albumin at the levels of maximum binding capacities. It is also considered possible that the presence of the much greater percentage of hydrocortisone in free form is a means of making this hormone more readily available to a greater variety of reactive tissues.

It is realized that any discussion on the physiological significance of the present findings must include the role of other serum protein components in the binding of steroid hormones. This problem is now being investigated by a more suitable procedure.

SUMMARY

Ultracentrifugal and electrophoretic studies demonstrated that one third to one fifth of hydrocortisone-4-C^{14} was free in the presence of human serum albumin. Up to two thirds were found to be bound to albumin. Almost the total amount of progesterone-4-C^{14} (99%) was attached to albumin. A small but definite portion (1%) of this steroid was present in free form. The hormone concentrations employed were physiological and constituted only a fraction of one per cent of the maximal binding capacities of serum albumin for these steroids.

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ANTI-GONADOTROPHIC ACTIVITIES OF QUINONES AND RELATED COMPOUNDS

FRED ROSEN AND NATHAN MILLMAN

with the technical assistance of Roger Homm

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THE normal reproductive processes in experimental animals are reported to be altered markedly by administration of 2,6-dimethylhydroquinone (DMHQ), a possible constituent of the pea *Pisum sativum* Linn. (Sanyal, 1952), and by extracts of various species of *Lithospermum* (Cranston, 1945; Zahl, 1948; Noble, Plunkett and Taylor, 1950). A crude tea prepared from the latter plant has been used by the American Indian in the past as an oral agent to achieve temporary sterility (Train, Henrichs and Archer, 1941). Inclusion of *Pisum sativum* Linn. in the diet of rats was found to induce sterility (Nag and Pain, 1935–36), while injections of the extracted oil produced constant estrus (Sanyal, 1950). The simultaneous injection of this oil and chorionic gonadotrophin in the immature rat resulted in the negation of the usual gonadal response induced by the hormone alone (Sanyal, 1950). Similarity in the anti-gonadotrophic activity of these materials of diverse botanical origin is evident in their ability, upon *in vitro* mixture with pregnant mare serum gonadotrophin (PMS), to interfere with the usual ovarian stimulating effects (Noble and Graham, 1953; Graham and Noble, 1955).

These observations are of profound significance in studies of the control of conception by materials of biological or synthetic origin. The object of these studies was to examine in more detail the optimum conditions and structural specificity required for the inactivation of PMS and to determine the effects of various anti-PMS compounds on the estrus cycle of the rat.

METHODS

Anti-Gonadotrophic Assay

The test substance was dissolved or suspended homogeneously in 0.3 ml. 0.1M phosphate buffer of proper pH, and this mixed with 100 I.U. of PMS[1] in 0.2 ml. of 0.5% phenol. The pH was taken at this point, adjusted if necessary and the mixture incubated at 37° C. Incubation time, pH, and concentration of the test agent were varied to determine the optimal conditions for the reaction.

Following incubation, the solution was injected subcutaneously into immature female rats weighing 40–75 grams. Seventy-two hours after injection, the animals were autopsied

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[1] "Equinex" (Ayerst, McKenna and Harrison).

and the ovaries and uterus were removed, cleaned, and weighed; the time required to dissect and weigh the organs was about 5 minutes. The uterine weights were not affected particularly by the various anti-PMS compounds tested and thus were omitted from this report.

Estrous Cycle Effect

Mature virgin rats weighing 175–225 gm. were used to evaluate the effect of various compounds on the estrous cycle. Groups of at least 3 animals were used for each compound tested. The rats were housed in air conditioned and temperature-controlled rooms, maintained on Purina Chow, and the test substance administered daily. Vaginal smears were made daily during the 2–4 week test period.

RESULTS

The *in vitro* inactivation of PMS by DMHQ is dependent upon a number of environmental conditions. Noble and Graham (1953) had suggested

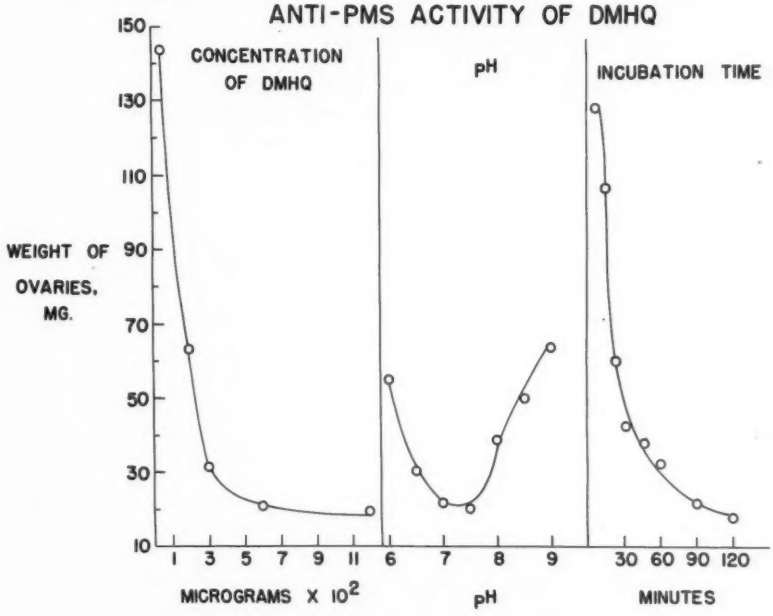

FIG. 1. Optimum conditions for inhibition by DMHQ of ovarian weight increase effect of PMS

that the reaction may be carried out at about pH 7.5, but buffered systems were not employed. When the reactions were carried out with 600 μg. of DMHQ against 100 I.U. PMS in systems buffered with 0.1M phosphate, optimum inactivation occurred between pH 7.0 to 7.5, as shown in Figure 1. In more acid or alkaline ranges the effect of the DMHQ is largely nullified, and the ovarian weight increase approached that obtained by uninhibited PMS itself.

min. A small but definite portion (1%) of this steroid was present in free form. The hormone concentrations employed were physiological and constituted only a fraction of one per cent of the maximal binding capacities of serum albumin for these steroids.

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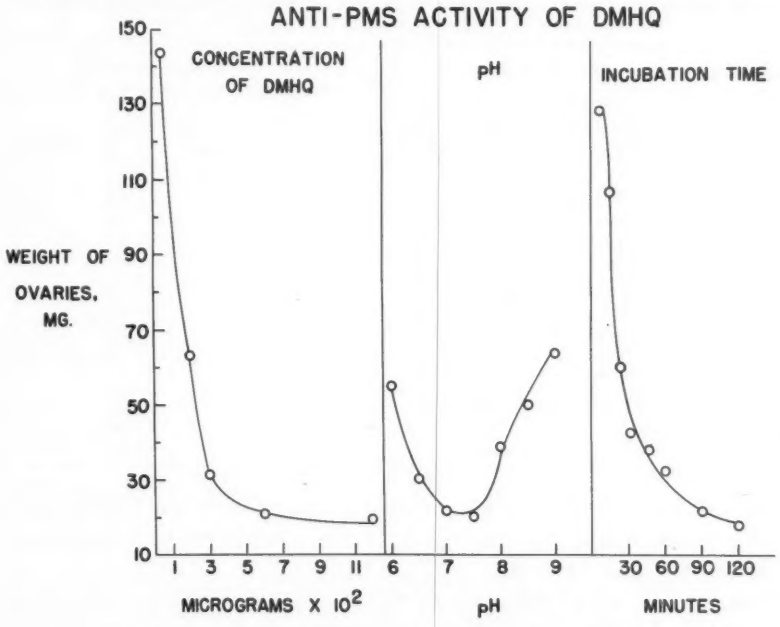

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A second prime factor in the inhibition phenomenon appeared to be the length of the incubation period. It was apparent from the work cited above that complete inhibition of PMS could be obtained by as little as 10 μg. of DMHQ if the incubation period was extended to 24 hours. In our *in vitro* systems containing 600 μg. of DMHQ at pH 7.5, 75% inhibition of 100 I.U. PMS occurred within the first 30 minutes of incubation, as seen in Figure 1. However, another 90 minutes were required to complete the inhibition.

These differences in the effect of concentration led us to determine the optimum concentration for inhibition in our system. From Figure 1 it may be seen that maximum inhibition was obtained with 600 μg. DMHQ, completely preventing the ovarian stimulation of 100 I.U. PMS. Although

TABLE 1. ANTI-PMS ACTIVITY OF QUINONES AND RELATED COMPOUNDS
All assays in duplicate vs. 100 I.U. PMS; pH 7.5 with 0.1M phosphate buffer. DMHQ conc. 0.6 mg.; Vit. E, 2.0 mg.; all others 1.0 mg.

Agent	Ovarian weight, mg.
None (PMS alone)	127
2,6-dimethylhydroquinone (DMHQ)	21
DMHQ plus Vit. E (α-tocopherol)	31
2-methyl-1,4-naphthoquinone	132
sodium gentisate	145
1,4-naphthoquinone	62
gallic acid	20
phenylquinone	20
catechol	20
pyrogallic acid	37
phloroglucinol	108
resorcinol	125
hexylresorcinol	25
hydroquinone	15
chlorohydroquinone	17
hydroquinone, monomethyl ether	114
3,4-dihydroxyphenylalanine	26
quercetin	126
catechin	133

Vitamin E has been reported to neutralize the activity of DMHQ on chorionic gonadotrophin (Sanyal, 1952), it was completely without effect in our anti-PMS assay system.

Other derivatives of quinones, as well as some of dihydroxybenzoic acid, are capable of PMS inactivation, as pointed out by Noble and Graham (1953, 1954). Our data on such compounds are presented in Table 1, and largely corroborate these reports. There are some significant differences, however, when the reactions are carried out under our conditions. We have been unable to demonstrate activity of 2-methyl-1,4-naphthoquinone or of sodium gentisate.

In addition to the compounds listed in Table 1 numerous quinones and compounds varying in number and relationship of hydroxyl groups have been tested as inhibitors of PMS. These were examined at levels of 1–3

mgm. in the presence of 100 I.U. of PMS. Tannic acid and 2,3,4-trihydroxydecanophenone limited ovarian weight increases to less than 50 mg., exhibiting considerable activity. However, all of the following compounds were without activity: 2,6-dimethylhydroquinone monobenzoate; 2,6-dimethoxybenzoquinone; tetrahydroxyquinone; gallein; citrazinic acid; carminic acid; meconic acid; quinic acid; xanthurenic acid; coumarin; inositol; polyporic acid; fluorescein; mucic acid; 3-hydroxyflavone; thymol.

The inhibition of gonadotrophin by DMHQ and related substances prompted us to determine whether this effect might be evident in *in vivo* systems as well. To this end we examined the effects of these agents on the rat estrous cycle. Table 2 summarizes the data on animals receiving 10 mg. of the test material by various routes of administration. Vaginal smears indicative of estrus or diestrus, lasting for approximately 9 days,

TABLE 2. EFFECTS OF ANTI-GONADOTROPHIC COMPOUNDS ON RAT ESTROUS CYCLE
All preparations at pH 7.5; all doses at 10 mg./day except as noted

Compound	No. of rats	Route of administration	No. of days on test	Effect on cycle	Days until inhibition of cycle	No. of days cycle inhibited
DMHQ	6	i.p.	19	Estrus (3) Diestrus (3)	6	8
	6	oral	25	None	—	—
	6	oral*	28	None	—	—
	6	s.c.	19	Diestrus	6	8
DMHQ plus 10 mg. Vit. E acetate	6	s.c.	18	Diestrus	6	12
Phenylquinone	3	i.p.	12	None	—	—
Hydroquinone	3	s.c.	11	Diestrus	3†	5
Gallic acid	4	s.c.	14	None	—	—
Hexylresorcinol	4	s.c.	14	None	—	—
1,4-naphthoquinone	4	s.c.	14	None	—	—

* 25–35 mg./kg. body weight.
† Only 2 of 3 animals.

were noted in all rats given DMHQ parenterally. However, when the same quantity of DMHQ was given by stomach tube, or when much larger amounts were included in the diet (2.0 grams/kilogram of Purina Chow), normal cycling was observed. Vitamin E did not interfere with the anti-estrous action of DMHQ as one might have expected from Sanyal's (1953) report that this vitamin could reverse the inhibitory effects of DMHQ.

With the exception of DMHQ and hydroquinone, various compounds which were previously found to be excellent inhibitors of PMS failed to affect the cycle during a prolonged period of parenteral treatment. However, as indicated in Table 2, the cycles were re-established after 7–12 days of stasis, even when the active compounds continued to be administered daily.

DISCUSSION

Reproducible results could be achieved for the anti-gonadotrophin assay when the reaction was carried out in the presence of a pH 7.5 phosphate buffer and incubated 2 hours at 37° C. Inhibitors have been found among hydro-, benzo-, and naptho-quinones, and polyhydroxy derivatives of benzene and benzoic acid. Among these groups, however, the specific activity of the agent is determined by its structure. For example, the relative positions of the two or more hydroxyl groups on a benzene nucleus will influence activity (Table 1). Thus catechol (1,2-dihydroxybenzene) and pyrogallic acid (1,2,3-trihydroxybenzene) are excellent inhibitors, whereas resorcinol (1,3-dihydroxybenzene) and phloroglucinol (1,3,5-trihydroxybenzene) are inactive. These data would indicate the necessity of 'ortho' relationship of the hydroxyls for activity. That this exclusion of activity for 'meta' relationship may be modified by other substituents is seen from the activity of hexylresorcinol.

Structural requirements in another group are seen in the finding that hydroquinone and 2-chlorohydroquinone were equally good inhibitors of PMS, but that modification of one of the hydroxyl groups by conversion to the methyl ether completely destroyed any activity. In another series 3,4-dihydroxyphenylalanine (DOPA) inhibited PMS, but compounds bearing some structural resemblance to it, quercetin and catechin, failed to influence PMS function. To this point, these data indicate a correlation of structure to activity and this correlation may become more distinct as the number and diversity of the agents tested increases. This information may then permit the prediction of the type of compound which may be expected to inactivate PMS in the *in vitro* reaction.

It does not follow, however, that compounds exhibiting such *in vitro* modification of gonadotrophic potency necessarily possess *in vivo* ability to do the same. Indeed, of several potent inhibitors, only DMHQ and hydroquinone affected the rat estrous cycle when they were given parenterally (Table 2); Noble and Graham (1953) had observed this effect for DMHQ given subcutaneously. The failure of the other substances to modify the estrous cycle may lie in unexplored factors such as metabolic disposition, detoxification rates and processes, and the influence of the routes of administration. Indeed, the two compounds which influenced the cycle upon daily parenteral injection were without any effect when administered orally in large amounts.

The results summarized in Table 2 show that DMHQ and hydroquinone given parenterally required an average induction period of 6 and 3 days respectively before the cycle was interrupted. With DMHQ the animals remain in estrus or diestrus for about 8 days or a period equal to two normal cycles. Following this period, and despite the fact that the animals continued to receive the agent, normal cycling resumed in all rats. Those receiving DMHQ plus Vitamin E remained in diestrus 12 days at which

time they were sacrificed. The cycle was interrupted for only 5 days with subcutaneous hydroquinone, after which normal cycling was observed. Thus, it would appear that these experimental animals become refractory to the effects of these compounds.

SUMMARY

The optimum conditions for determining the anti-PMS activity of 2,6-dimethylhydroquinone (DMHQ) were studied. Other agents among the quinones and from several classes of polyhydroxy compounds had varying inhibitory activity by this *in vitro* test, depending upon the individual chemical structure. Only DMHQ and hydroquinone, however, affected reproductive processes in *in vivo* tests upon injection, as shown by rat estrous cycle changes. Neither of these were active when given orally. Upon continued parenteral administration rats became refractory to these two compounds and resumed cycling.

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RESPONSE OF THE BLOOD CORPUSCLES IN HYPOPHYSECTOMIZED RATS TO IRRADIATION WITH X-RAY[1]

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SOME attempts have been made to demonstrate the influence of hormones on the sensitivity of animals to irradiation and on their capacity to recover from damage induced by it. Evidence concerning the part played by the anterior hypophysis is conflicting. Although Patt, Swift, Tyree, and Straube (1948) found that hypophysectomy increases the sensitivity of rats to irradiation with X-ray, Finerty, Binhammer and Schneider (1953) concluded from experiments utilizing the parabiosis technique, that secretions of the hypophysis and of its dependent glands are not critical factors in recovery.

There are several reasons why the hypophysectomized animal might be less resistant to X-irradiation. Pituitary ablation causes numerous structural and functional deficiencies in the alimentary tract (Baker and Abrams, 1954, 1955) which could lessen the capacity of an animal to withstand the early gastro-intestinal phase of the radiation syndrome.

Pertinent hematological changes also occur after hypophysectomy. An anemia ensues which is characterized by a reduction in the reticulocyte percentage (Stewart, Greep and Meyer, 1935), erythrocyte count, hemoglobin concentration (Meyer, Stewart, Thewlis and Rusch, 1937), hematocrit (Crafts, 1953a), and total erythrocyte volume (Berlin, Van Dyke, Siri and Williams, 1950). The bone marrow undergoes involution and, under some conditions, the anemia is of a microcytic, hypochromic type (Crafts, 1946). The manner in which the anterior hypophysis affects the formation of erythrocytes is in dispute. Crafts (1953b) holds that this action is mediated by the adrenal cortex and thyroid glands while Contopoulos *et al.*, (1953, 1954a, b) maintain that the pituitary gland secretes an erythropoietic hormone which acts directly on the bone marrow.

Hypophysectomy might influence recovery from irradiation because it

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alters the number of circulating leucocytes. Crafts (1941) observed a slight leucocytosis and Gordon (1954), a leucopenia. This report will demonstrate that in hypophysectomized rats a significant recovery occurs in the number of circulating blood cells after their formation has been suppressed by total body X-irradiation.

PROCEDURE

Adult female Sprague-Dawley rats were divided into the following groups: (a) 5 were non-operated and fed *ad libitum*; (b) 7, non-operated and pair-fed; (c) 7, non-operated and irradiated; (d) 14, hypophysectomized and (e) 21 were hypophysectomized and irradiated. Following hypophysectomy all rats were placed in individual cages. Excepting those in group (a) all non-operated animals were pair-fed against the hypophysectomized rats during the first 21 days, i.e., the average daily food intake of the hypophysectomized rats was given on the succeeding day to each rat in the other groups. The diet consisted of Purina Laboratory Chow supplemented twice weekly with greens, oranges, and a vitamin concentrate. On day 21, groups (c) and (e) were given 500 r of total body X-radiation. Eight rats were treated simultaneously while held in a disc-shaped Lucite container, which was subdivided into 8 radially divided compartments. The conditions for irradiation were as follows: 200 KV, $\frac{1}{2}$ mm. Cu plus 1 mm. Al filtration, 110 cm. from the center line of the X-ray tube to the table on which the animal container rested, and a dose rate of about 11 r/minute. The X-ray intensity was calibrated with a Victoreen condenser r-meter. The dose rate was indicated by a Victoreen rate meter and held constant by varying the X-ray tube current. Beginning the day after irradiation all rats except those in group (a) were pair-fed against the hypophysectomized irradiated rats since these consumed the least amount of food.

Blood studies were carried out on the day before irradiation and on every seventh day thereafter for 11 weeks. The blood was secured by incising a vein of the tail after placing the member in warm water. A Neubauer chamber was used for erythrocyte and total leucocyte counts. Hemoglobin concentration was determined with a Sahli-Hellige hemometer. The hematocrit was determined on oxalated flood which was placed in Van Allen tubes and centrifuged at 1610 r.p.m. for 30 minutes in a size 1, type SB, International centrifuge. Two blood films were made, one being stained with Wright's stain and the other with brilliant cresyl blue and Wright's stain for reticulocytes. One thousand erythrocytes were counted in order to determine the percentage of reticulocytes. The mean diameter of erythrocytes was derived from measurement of 100 cells with an ocular micrometer prior to irradiation and at 2, 4 and 11 weeks afterwards. The probability of error was determined by use of the Student-Fisher t formula except when the variance ratio was significantly high. In the latter case, the following test (Welch) was used:

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\frac{L_1}{N_1^2(N_1 - 1)} \frac{L_2}{N_2^2(N_2 - 1)}}}.$$

Completeness of hypophysectomy was verified by microscopic examination of the serially sectioned pituitary capsules. If fragments of the pars distalis were present, the animal was eliminated from the experiment. The body weights at the time of hypophysectomy of the rats in groups (d) and (e) (Table 1) approximated those of groups (b) and (c) much more closely than indicated because the hypophysectomized rats were weighed one or two days after the operation rather than before. The resultant loss in weight amounted to several grams.

TABLE 1. MEAN BODY WEIGHTS (GM.) OF THE RATS

Group	At hyp.	At irrad.	At autopsy
a) Non-hyp. Ad lib.	181 ± 15	205 ± 11	251 ± 11
b) Non-hyp. Pair-fed	175 ± 3	195 ± 11	211 ± 12
c) Non-hyp. Irrad.	172 ± 12	196 ± 13	209 ± 14
d) Hyp.	159 ± 7	154 ± 7	154 ± 9
e) Hyp. Irrad.	166 ± 11	161 ± 8	154 ± 12

The following abbreviations are used in all tables: ± = standard deviation, hyp. = hypophysectomy, ad lib. = *ad libitum*, irrad. = irradiated.

OBSERVATIONS

Erythrocyte count. Reduction in the amount of food eaten by the non-hypophysectomized rats to that consumed by the hypophysectomized-irradiated animals did not modify the number of circulating erythrocytes (Groups a and b, Table 2). After hypophysectomy the mean number of erythrocytes was reduced from 8.87 ± standard deviation .80 million per cu. mm. 3 weeks after the operation (0 weeks, Group d) to 6.38 ± .54 four weeks later, the decrease being significant ($P = <.001$). At the latter time, the mean for this group was also lower than that of the non-hypophysectomized group (b) ($P = <.01$). In the later weeks of the observation period, a gradual increase occurred in the erythrocyte count of the hypophysectomized rats but at the end of the experiment, the mean count remained lower than that of the non-hypophysectomized group (b) ($P = <.02$).

Irradiation of non-hypophysectomized rats (Group c, Table 2) reduced the number of erythrocytes from 7.84 ± .92 million per cu. mm. before irradiation to 5.96 ± 1.04 two weeks later ($P = <.001$). Although recovery occurred, the count did not return the level shown by the non-irradiated, non-hypophysectomized rats (Group b) during any single week of the observation period. In the hypophysectomized-irradiated rats (Group e) a still greater depression occurred, reaching a mean of 4.32 million per cu. mm. at the fourth week. This value is significantly different from that for the hypophysectomized controls (Group d) at the same week ($P = <.001$), and that of the non-hypophysectomized, irradiated rats (Group c) at the

TABLE 2. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN NUMBER OF ERYTHROCYTES (MILLION/CU. MM.)

Group	Weeks after irradiation											
	0	1	2	3	4	5	6	7	8	9	10	11
a) Non-hyp. Ad lib.	8.53 ± .87	7.38 ±1.0	7.74 ± .61	7.92 ± .54	7.90 ± .40	8.22 ± .40	8.22 ± .33	8.50 ± .84	8.24 ± .62	8.58 ± .43	8.48 ± .71	8.14 ± .48
b) Non-hyp. Pair-fed	8.50 ± .83	7.77 ± .79	7.53 ± .76	8.02 ± .70	7.67 ± .86	8.30 ± .92	8.22 ± .50	8.15 ± .31	7.57 ± .64	7.91 ± .54	8.04 ± .42	8.23 ± .43
c) Non-hyp. Irrad.	7.84 ± .92	7.32 ± .73	5.96 ±1.0	6.08 ±1.3	7.55 ±1.0	7.53 ± .50	7.47 ± .61	7.74 ± .87	7.39 ± .60	7.52 ± .35	7.48 ± .38	7.61 ± .62
d) Hyp.	8.87 ± .80	7.18 ± .99	7.17 ± .84	7.11 ±1.0	6.38 ± .54	6.93 ± .52	6.85 ± .67	6.79 ± .85	6.84 ±1.5	7.06 ± .72	7.26 ± .50	7.26 ± .68
e) Hyp. Irrad.	8.37 ± .67	7.32 ±1.3	5.95 ± .95	4.54 ±1.1	4.32 ± .82	5.14 ± .88	5.42 ± .63	5.30 ± .50	5.66 ± .76	5.74 ± .48	6.05 ± .61	6.17 ± .64

second week (P = <.01). Recovery occurred but at the eleventh week the mean count was still lower than that for the hypophysectomized non-irradiated rats (P = <.001).

Hemoglobin. In general, the changes in hemoglobin concentration paralleled those which occurred in the number of erythrocytes. Hypophysectomy reduced the mean hemoglobin concentration from 16.2 ± 1.8 gm./100 cc. at 3 weeks after the operation (0 weeks, Group d, Table 3) to 12.2 ± 0.6 five weeks later (P = <.001). Subsequently, a small but significant increase occurred so that at 14 weeks (11 weeks after irradiation) the mean was 13.5 ± 1.4 (P = <.05).

Irradiation of non-hypophysectomized rats reduced the hemoglobin concentration from 16.1 ± 0.8 gm./100 cc. to 12.3 ± 2.1 at the second week (Group c, Table 3), the decrease being significant (P = <.01) as was also the difference between this group and the non-hypophysectomized, non-irradiated rats (P = <.01) at the second week. By the fourth week, the

TABLE 3. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN HEMOGLOBIN CONCENTRATION (GM./100 CC.)

Group	Weeks after irradiation											
	0	1	2	3	4	5	6	7	8	9	10	11
a) Non-hyp. Ad lib.	15.3 ±1.2	14.9 ±0.9	15.6 ±2.0	15.9 ±1.8	16.2 ±0.5	16.0 ±0.6	16.1 ±0.8	17.0 ±1.7	16.3 ±1.8	15.8 ±1.9	15.3 ±0.7	15.5 ±1.1
b) Non-hyp. Pair-fed	16.3 ±1.1	15.5 ±1.1	15.9 ±1.0	16.2 ±1.7	15.9 ±1.0	17.0 ±1.2	16.2 ±1.6	16.9 ±1.6	15.7 ±0.9	16.0 ±0.8	15.0 ±1.4	15.3 ±1.6
c) Non-hyp. Irrad.	16.1 ±0.8	14.3 ±1.2	12.3 ±2.1	13.0 ±2.2	15.7 ±0.9	16.0 ±2.1	15.3 ±1.4	15.8 ±0.7	15.2 ±1.7	16.2 ±1.7	15.0 ±0.8	14.9 ±1.2
d) Hyp.	16.2 ±1.8	13.8 ±1.8	13.7 ±1.7	12.8 ±1.3	12.8 ±0.6	12.2 ±0.6	12.8 ±1.0	13.1 ±1.0	13.1 ±0.8	13.2 ±1.3	13.3 ±1.5	13.5 ±1.4
e) Hyp. Irrad.	15.9 ±1.5	14.4 ±1.6	11.4 ±2.3	8.5 ±1.9	9.0 ±2.0	10.4 ±1.4	11.4 ±0.9	11.6 ±0.9	11.3 ±1.3	11.8 ±1.4	12.1 ±1.3	12.2 ±1.1

hemoglobin concentration in the non-hypophysectomized, irradiated rats had returned to the level shown by the non-irradiated, non-hypophysectomized controls, the differences between groups b and c no longer being significant.

Irradiation of hypophysectomized rats reduced the hemoglobin concentration to 8.5 ± 1.9 gm. % at the third week after irradiation (Group e, Table 3). It then returned to 12.2 ± 1.1 at the eleventh week. Both the drop and later rise were significant at less than the 0.1% and 1% levels of confidence, respectively. Although the level of hemoglobin concentration continued lower than that of the hypophysectomized controls (Group d) at the eleventh week after irradiation, the difference between the means for the two groups was not significant (P = >.05).

Hematocrit. Hypophysectomy reduced the hematocrit from a mean of 50.0 ± 2.4% at 3 weeks after the operation (0 weeks, Group d, Table 4) to 38.8 ± 2.6 four weeks later (P = <.001). Although there followed a slight rise to 42.0 ± 5.1 at the end of the experiment the mean remained lower

than at the beginning of the observation period (P = <.01).

Irradiation of non-hypophysectomized rats reduced the hematocrit from 49.8±3.9% to 38.0±7.2 two weeks later (Group c, Table 4) (P = <.01). The hematocrit returned to the level of the non-hypophysectomized, non-irradiated rats (Group b) at 6 weeks and remained at this level for the remainder of the experiment. In hypophysectomized rats (Group e) the lowest mean hematocrit, 27.6±10% appeared 3 weeks after irradiation, this drop being significant (P = <.001). The hematocrit increased to 43.3±5.0 at the eleventh week (P = <.001) which was comparable with the value for the hypophysectomized, nonirradiated controls (Group d).

Diameter of erythrocytes. The average of all of the means of the diameters of erythrocytes for the non-irradiated, non-hypophysectomized groups (a and b, Table 5) was 6.22μ. The greatest difference in these means, which occurred at 4 weeks, was not significant (P = >.4). Creskoff, Fitzhugh

TABLE 4. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN HEMATOCRIT (% ERYTHROCYTES)

Group	Weeks after irradiation											
	0	1	2	3	4	5	6	7	8	9	10	11
a) Non-hyp. Ad lib.	51.0 ±5.1	46.7 ±3.0	48.6 ±3.9	49.8 ±5.8	50.0 ±3.4	52.0 ±4.6	50.8 ±7.2	45.6 ±6.7	49.2 ±1.0	51.2 ±2.7	49.0 ±2.4	52.1 ±2.1
b) Non-hyp. Pair-fed	50.0 ±7.2	49.3 ±5.6	46.6 ±3.6	51.2 ±4.7	49.7 ±2.1	50.3 ±3.6	45.1 ±5.1	48.5 ±3.5	49.8 ±4.5	48.0 ±2.7	48.8 ±2.1	50.7 ±4.9
c) Non-hyp. Irrad.	49.8 ±3.9	47.5 ±5.4	38.0 ±7.2	43.4 ±9.9	47.9 ±2.8	48.8 ±5.1	51.9 ±4.3	46.9 ±4.2	48.5 ±4.6	47.1 ±2.5	49.8 ±3.9	48.5 ±2.2
d) Hyp.	50.0 ±2.4	45.6 ±3.7	47.2 ±6.4	42.9 ±5.1	38.8 ±2.6	43.0 ±8.5	44.8 ±4.7	42.2 ±3.6	45.0 ±5.5	40.4 ±5.0	40.3 ±8.1	42.0 ±5.1
e) Hyp. Irrad.	51.6 ±3.3	48.6 ±7.1	39.1 ±7.2	27.6 ±10	31.5 ±8.6	37.4 ±6.8	39.0 ±6.3	39.0 ±1.6	40.2 ±5.5	42.0 ±4.9	41.0 ±5.5	43.3 ±5.0

and Farris (1942) gave 6.3μ as the mean for adult female rats. Hypophysectomy did not induce microcytosis as has been reported to occur (Crafts, 1946); rather, the diameter of the erythrocytes was somewhat greater than in the non-hypophysectomized rats.

Although the mean diameter in the non-hypophysectomized irradiated group (c, Table 5) increased from 6.36μ at 0 weeks to 6.50μ at the eleventh week, indicating the appearance of macrocytosis, the difference was not significant (P>.2). On the other hand, after hypophysectomy (Group e) macrocytosis was induced, the mean diameter increasing from 6.40μ before irradiation to 6.74μ eleven weeks later (P = <.001). The latter value was also greater than that for the non-hypophysectomized, irradiated rats at the eleventh week (P = <.05). The presence of macrocytosis after irradiation of hypophysectomized rats and the failure of hypophysectomy alone to cause microcytosis were confirmed by determination of the mean corpuscular volume.

Reticulocytes. Three weeks after hypophysectomy (0 weeks, Table 6) the

TABLE 5. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN DIAMETER OF ERYTHROCYTES (μ)

Group	No. rats	Weeks after irradiation			
		0	2	4	11
a) Non-hyp. Ad lib.	5	6.28 ± .21	6.29 ± .13	6.38 ± .13	6.30 ± .20
b) Non-hyp. Pair-fed	6	6.08 ± .07	6.14 ± .19	6.16 ± .21	6.19 ± .25
c) Non-hyp. Irrad.	6	6.36 ± .24	6.25 ± .35	6.48 ± .22	6.50 ± .13
d) Hyp.	12	6.41 ± .16	6.44 ± .09	6.40 ± .22	6.40 ± .19
e) Hyp. Irrad.	17	6.40 ± .17	6.39 ± .20	6.62 ± .31	6.74 ± .22

reticulocyte percentage was 0.69 ± .36 and 0.26 ± .21 in groups d and e, respectively. On the other hand, in the 3 non-hypophysectomized groups (a, b and c), the percentages were 1.9 ± .50, 1.98 ± 1.03 and 1.67 ± .88. These differences demonstrate that formation of new erythrocytes was suppressed by removal of the pituitary gland and confirm the observations of many other investigators. From the third through the sixth week after hypophysectomy (Group d, weeks 0 to 6, Table 6) a rise occurred in the reticulocyte percentage, the difference between the means at these two times being significant ($P = <.001$). A transitory spontaneous recovery in erythrocyte production was indicated in spite of the absence of the pituitary gland.

Irradiation of non-hypophysectomized rats (Group c, Table 6) caused a statistically insignificant drop in the percentage of reticulocytes to 0.60 ± .77 at the first week. The percentage increased to 3.58 ± 1.67 at the third week. The difference between these means is significant at the 1% level. During the subsequent weeks, the number of reticulocytes tapered off to approximately the level of the non-hypophysectomized, non-irradiated controls.

In the hypophysectomized rats (Group e) irradiation also caused an

TABLE 6. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN PERCENTAGE OF RETICULOCYTES

Group	Weeks after irradiation							
	0	1	2	3	4	5	6	11
a) Non-hyp. Ad lib.	1.90 ± .50	1.74 ± .73	1.90 ± .42	1.84 ± .67	1.18 ± .43	2.06 ± .27	1.46 ± .82	1.70 ± .87
b) Non-hyp. Pair-fed	1.98 ±1.0	0.92 ± .41	1.82 ± .64	1.76 ± .57	1.32 ± .39	1.73 ± .81	2.20 ± .90	1.80 ± .78
c) Non-hyp. Irrad.	1.67 ± .88	0.60 ± .77	3.41 ±2.3	3.58 ±1.7	2.22 ±1.2	2.27 ± .28	2.03 ± .64	1.32 ± .50
d) Hyp.	0.69 ± .36	0.98 ± .97	0.98 ± .98	0.98 ± .59	1.78 ± .95	2.08 ± .66	2.28 ± .56	1.11 ± .75
e) Hyp. Irrad.	0.26 ± .21	0.12 ± .25	0.51 ± .46	2.77 ±1.7	6.44 ±4.1	5.47 ±3.6	3.21 ±1.2	1.16 ± .69

insignificant fall in the percentage of reticulocytes to a mean of $0.12 \pm .25$ at the first post-irradiation week. However, this mean was significantly lower than that for the non-hypophysectomized irradiated group (c) at the first week ($P = <.05$). By the fourth week a significant ($P = <.001$) increase had occurred in the reticulocytes of the hypophysectomized, irradiated rats (Group e) reaching a mean of 6.44 ± 4.05. This rebound was considerably greater than that which characterized the non-hypophysectomized, irradiated rats. Plotting the lowest erythrocyte count shown by each animal in group (e) against its highest reticulocyte count, indicated that an inverse relationship existed between the two, although this was not clear in all cases. Thus, the higher reticulocyte counts exhibited by the hypophysectomized-irradiated rats during recovery from irradiation probably were related to the greater depression which had occurred in their erythrocyte production.

Leucocytes. Hypophysectomy caused a mild leucocytosis (Group d, Table 7) which at the time of irradiation was not significant as compared with the number of leucocytes in the non-hypophysectomized group (b) ($P = >.05$). After irradiation the lowest mean count occurred at one week in the non-hypophysectomized group (c) and at 2 weeks in the hypophysectomized group (e), these means being of comparable magnitude (about 2,400). In the former group, the count returned at the eleventh week to the level for the rats which were neither irradiated nor hypophysectomized (Groups a and b). The mean number of leucocytes in the hypophysectomized-irradiated rats (Group e) increased to 13,600 at the eleventh week which was less than the count for the non-irradiated, hypophysectomized rats (16,900) ($P = <.05$) but somewhat more than that which characterized the non-hypophysectomized-irradiated group (c) ($P = >.05$).

TABLE 7. THE EFFECT OF HYPOPHYSECTOMY AND IRRADIATION ON THE MEAN NUMBER OF LEUCOCYTES (THOUSANDS/CU. MM.)

Group	Weeks after irradiation											
	0	1	2	3	4	5	6	7	8	9	10	11
a) Non-hyp. Ad lib.	15.8 ±2.9	12.2 ±2.7	17.9 ±5.2	12.7 ±2.4	16.4 ±2.6	14.4 ±3.1	12.8 ±2.7	12.1 ±1.9	12.1 ±2.5	11.1 ±3.7	12.0 ±5.0	9.5 ±1.7
b) Non-hyp. Pair-fed	17.9 ±6.9	13.4 ±5.7	13.2 ±3.5	15.4 ±4.4	16.4 ±3.0	16.9 ±1.5	14.0 ±2.7	14.3 ±4.2	15.1 ±2.3	12.1 ±1.6	11.4 ±2.0	11.5 ±2.1
c) Non-hyp. Irrad.	16.3 ±3.4	2.4 ±1.4	2.8 ±1.3	5.5 ±1.9	8.9 ±3.0	10.4 ±3.4	10.0 ±4.0	11.0 ±1.9	9.7 ±2.5	9.5 ±2.5	10.4 ±4.3	11.3 ±3.3
d) Hyp.	23.1 ±6.5	20.2 ±4.2	20.9 ±4.6	21.6 ±3.9	19.9 ±3.9	18.7 ±5.6	17.4 ±3.4	17.4 ±3.3	16.2 ±4.0	16.6 ±3.8	16.7 ±2.6	16.9 ±3.9
e) Hyp. Irrad.	24.9 ±6.6	4.2 ±4.0	2.4 ±9.3	4.2 ±1.8	8.2 ±2.0	11.2 ±4.2	11.3 ±3.3	11.9 ±3.9	13.3 ±3.7	13.3 ±2.7	12.2 ±2.5	13.6 ±3.7

DISCUSSION

Following an early depression in erythropoiesis in the hypophysectomized rats (Group d, Tables 2–5) some evidence of recovery was observed. This was revealed by gradual elevations in the erythrocyte count

and hemoglobin concentration which were observed between the seventh and fourteenth weeks after hypophysectomy. A less significant elevation in the hematocrit percentage occurred during this period also. One might assume that these changes were reflections of a reduction in plasma volume except that a concurrent elevation in the reticulocyte percentage was present. Similar observations have not been made by other workers and an explanation is not apparent. However, it is becoming clear that the anemia which follows hypophysectomy and ablation of other endocrine glands varies in severity according to the strain of rat under study (Gordon, 1954; Meineke and Crafts, 1955). Diet also modifies the degree of anemia (Gordon, 1954). This factor could not have been responsible for our observations which were made in several different experiments conducted at different times of the year, with the elevations in erythroid values being obtained rather regularly. Insofar as we are aware, the diet did not vary during the experiment. However, the rats did receive greens twice a week, which according to Gordon (1954), may modify the anemia.

The corpuscular changes induced by total body irradiation of non-hypophysectomized rats were similar to those summarized by Laurence, Dowdy, and Valentine (1948). It is clear that erythrocyte production was suppressed more severely by irradiation in the absence of the hypophysis. This was indicated by the greater fall which occurred in the erythrocyte and reticulocyte counts and in the hemoglobin concentration.

The primary objective of this investigation was to determine whether the blood cell-producing organs can recover from the suppressing action of irradiation in the absence of the hypophysis. If the production of erythrocytes by the bone marrow is dependent on stimulation by a pituitary erythropoietic hormone (Contopoulos *et al.* 1953), such recovery would seem improbable. To our knowledge, recovery of blood-forming organs in hypophysectomized animals from the effects of any other suppressing agent has not been demonstrated. However, Feigin and Gordon (1950) showed that erythroid tissue in hypophysectomized rats does respond to the *stimulation* induced by anoxia if the oxygen pressure is sufficiently low.

It is clear from our observations that the recovery of blood-forming organs from the effects of total body X-irradiation is not dependent upon the presence of the hypophysis. The formation of erythroid cells was restored in the hypophysectomized rats although this recovery was incomplete since the erythrocyte count and hemoglobin concentration did not return to the levels characteristic of the hypophysectomized, non-irradiated controls. Consideration should be given to the possibility that the erythrocyte count, hemoglobin concentration and hematocrit percentage rise during the recovery period because of a reduction in volume of the plasma. Although this may be a factor, significant activation of the erythroid tissue was demonstrated by the strikingly high reticulocyte counts

which were obtained in the hypophysectomized-irradiated rats.

The hematocrit of hypophysectomized-irradiated rats returned to the level of the non-irradiated hypophysectomized controls even though the number of erythrocytes failed to do so. This may be explained on the basis of the macrocytosis which resulted from irradiation of hypophysectomized rats. Erythrocyte enlargement may be related to an intrinsic change in the structure or metabolism of the erythrocyte since Megel and Gordon (1952) observed that the fragility of erythrocytes in hypophysectomized rats is reduced.

Other unpublished studies by our group support the contention of Patt *et al.*, (1948) that hypophysectomy increases the sensitivity of rats to total body irradiation with X-ray. Part of the explanation for this change probably lies in the impairment of the capacity of the erythroid tissue to recover as completely as when the hypophysis is present. The rapid return of the total leucocyte count to or above normal values suggests that from the standpoint of numbers, they were adequate to cope with the bacterial phase of the radiation syndrome. Lymphocytes were involved primarily in this recovery but our studies have not yet revealed the exact degree of participation by neutrophiles.

We have found that irradiated rats are more prone to die during the early gastro-intestinal phase of the radiation syndrome if they are hypophysectomized (Kent, Baker, Pliske, Van Dyke and Bethell, 1955). Their greater susceptibility appears to be related to the severe involution of certain components of the gastro-intestinal mucosa which follows pituitary ablation (Baker and Abrams, 1954, 1955). Thus, there are factors other than hematological which may account for the increased susceptiblity of hypophysectomized rats to irradiation.

SUMMARY

Three weeks after hypophysectomy, young adult female rats were given one dose (500 r) of total body X-irradiation. The ensuing depression in the erythrocyte and reticulocyte counts, hematocrit percentage and hemoglobin concentration was more severe than in the non-hypophysectomized, irradiated rats. During the 11-week period of observation, the erythrocyte count and hemoglobin concentration in hypophysectomized-irradiated rats returned toward the level of that shown by the non-irradiated hypophysectomized rats. The reticulocyte count and hematocrit percentage equalled or surpassed the values for the latter group. Macrocytosis resulted from irradiation in only the hypophysectomized rats. The total leucocyte count fell to the same level in both hypophysectomized and non-hypophysectomized rats but showed greater recovery in the hypophysectomized-irradiated animals. It was concluded that the capacity of the blood-forming tissues to recover from the damaging effects of X-irradiation is not completely dependent on the action of pituitary hormones.

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STUDIES ON *IN VITRO* PERFUSION OF BEEF AND DOG THYROID GLANDS USING RADIOIODINE[1]

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THE maintenance of the thyroid gland *in vitro*, by means of vascular perfusion with a medium containing radioactive iodide, might provide a means of evaluating the direct effect of various substances on thyroid function. Roche and associates (1951) perfused beef thyroids with beef serum containing radioactive iodide in order to prepare radioactive thyroglobulin. Under aseptic conditions the beef thyroid collected I^{131} for 24 hours. After 5½ hours of perfusion, radiothyroxine could be demonstrated in the thyroid.

The purpose of this study was to delimit the general applicability of *in vitro* perfusion of the thyroid to problems in thyroid physiology. The thyroid glands of the beef and dog were perfused under nonsterile conditions, and a survey was made of the *in vitro* effect of thyroid-stimulating and thyroid-inhibiting agents. Radioactive iodine was used to measure various aspects of thyroidal function.

MATERIALS AND METHODS

An anatomic survey and preliminary perfusion studies were made on the thyroid of several common laboratory animals and the cow. On the basis of the relatively large size of the thyroid and ease of cannulation, the beef and dog thyroids were selected for further study.

The thyroid glands from 28 male and two female mongrel dogs weighing 18 to 30 kg. were used. These dogs were fed Fromm dog food containing 0.5% iodized salt for at least 2 weeks prior to operation. The dogs were anesthetized with pentobarbital sodium and heparinized. Under nonsterile conditions, approximately 300 ml. of blood was collected and both lobes of the thyroid and a segment of the common carotid artery were removed.

The beef thyroids were obtained from a local abattoir.[2] Only one lobe of each beef thyroid was used for perfusion. Glucose citrate solution (Hechter *et al.*, 1953) was added to the beef blood at the time of collection when perfusion was to be carried out with whole blood. When serum was desired, the blood was allowed to clot. The blood or serum was stored at 4° C. for 1 to 4 days before use.

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[2] The authors wish to thank Mr. Harry R. Schneider for his co-operation in obtaining the beef thyroids from his abattoir.

Both the beef and dog thyroids were flushed with 0.9% solution of sodium chloride and perfused through the superior thyroid artery. The major accessory branches from the thyroid artery to the surrounding musculature were ligated. Perfusion of the thyroids was started 70–95 minutes after removal from the animals. During most of this interval the thyroids were kept cold.

The apparatus for either pulsatile or gravity perfusion was similar to that used by Hechter (1948) and by Hechter *et al.* (1953). The temperature of the perfusing medium was maintained between 37.5° C. and 38.5° C. The pressure used for all the dog-thyroid perfusions was approximately 100 to 110 mm. of mercury systolic and 60 to 70 diastolic. The thyroidal accumulation of I^{131} during the perfusion period was determined by either a scintillation counter or a Geiger-Müller counter placed directly over the thyroid. A decade scaler or a ratemeter was used to measure counts or counting rates. An initial background count was taken over the thyroid after it had been filled with perfusing medium. The per cent of the dose accumulated by the thyroid was determined by relating the radioactivity of the dissected thyroid to a pilot of the administered dose. In experiments with beef thyroids, the changes occurring in the radioactive content of the medium also were determined by a scintillation counter facing a constant volume of the medium.

Penicillin G sodium (Merck & Company, Rahway, New Jersey), 100 units per ml., and streptomycin (Merck & Company, Rahway, New Jersey), 100 μg. per ml., were added to all of the perfusing mediums. The concentration of carrier-free I^{131} in the mediums was 0.05 to 0.06 μc. per ml. The volume of medium was 500 to 700 ml. for the beef thyroid and 250 ml. for the dog thyroid. An initial sample of the medium was removed for analysis before perfusion was begun. No fortification of the medium with such factors as DPN, ATP or certain vitamines was done in these experiments.

Total blood I^{127} and serum or plasma $PBI^{\cdot 127}$ were determined by the method described by Barker (1948).[3] Trichloroacetic acid (T.C.A.)-precipitable I^{131} was determined in the thyroid and plasma or serum obtained from the initial and final sample of the medium. Column chromatography of thyroid tissue was done by the method described by Braasch, Flock and Albert (1954). The "radiothyroxine" content of the medium was determined by butanol extraction at a pH of 2 or lower of a $Zn(OH)_2$ precipitate of the plasma. At the end of the experiment, a portion of the thyroid was fixed in 10% formalin. Paraffin sections were made and stained with hematoxylin and eosin and in some instances with Brown Gram stain.

RESULTS

A. Beef Thyroid. 1. Pulsatile Perfusion with Citrated Blood.—The course of the collection, organic binding and release of I^{131} by the thyroid was determined in one experiment. The preparation consisted of one lobe of the thyroid and a rectangular block of underlying trachea and esophagus. The cut edges of the preparation were clamped with hemostats and the superior and inferior thyroid veins were cannulated. One-ml. samples were removed from the arterial, venous and seepage tubes at 15-minute intervals for the first hour and hourly thereafter for counting and for determining T.C.A.-precipitable I^{131}. The approximate perfusion pressure was 100 to 110 mm. of mercury systolic and 80 to 90 diastolic.

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[3] The authors are indebted to Dr. M. H. Power for the chemical determinations of the iodine content of the perfusates.

The thyroid lobe accumulated 77% of the dose of I^{131} in 6 hours (Fig. 1). A correction was made in the first part of the thyroid curve by lowering the initial observed value 2.5%, then gradually diminishing the correction and joining the thyroid curve at 90 minutes. This was an estimate of the correction for the radioactive blood in the thyroid initially since in this particular experiment an initial background count was not taken.[4]

The average efficiency of extraction of I^{131} by the thyroid was 14.3%

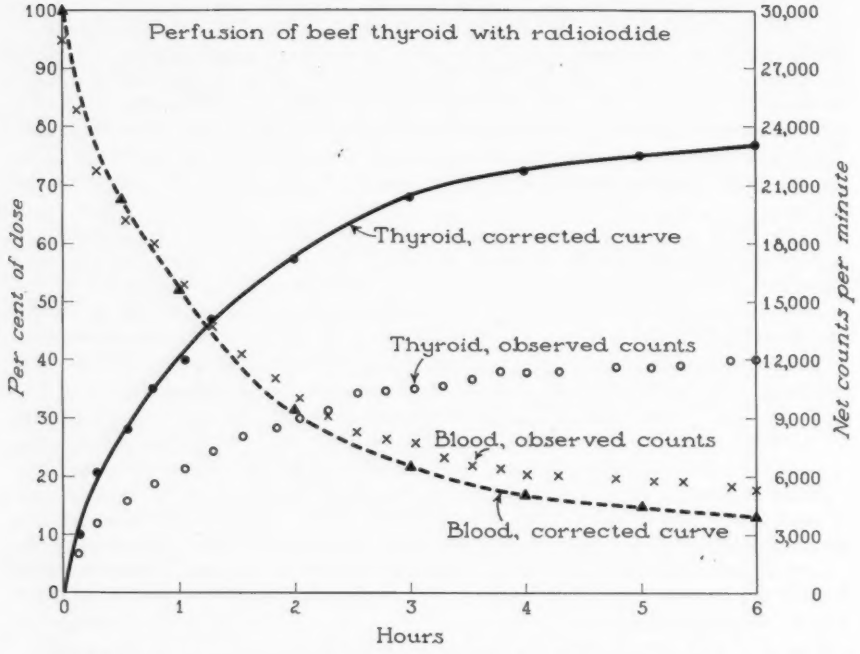

FIG. 1. Disappearance curve of I^{131} from the blood and the curve of accumulation of I^{131} by the thyroid. An explanation of the corrections made in the curves is given in the text.

(Table 1). Pochin (1950) estimated a mean efficiency of 20% for the normal human thyroid.

The venous return-flow rate decreased from 36.6 ml. to 8.4 ml. per minute in the first hour and then gradually decreased to 2.0 ml. per minute during the next 5 hours.

[4] Approximately 100 ml. of the circulating volume had been lost by hemorrhage and edema into the clamped tissue and by evaporation. This loss was distributed in equal proportions throughout the perfusion period and added to the loss removed as blood samples. The corrected circulating volume was then used to lower the observed values from the blood-counting system with a resultant corrected curve for the per cent of the dose remaining in the circulation.

A definite increase in the T.C.A.-precipitable I^{131} of the medium occurred after the first hour (Table 1). Further analyses of the initial and final samples of the medium revealed a decrease in the radioiodide fraction from 93.0% to 61.9%, an increase in the "radiothyroxine" fraction from 0.03% to 13.4% and an increase in the "radiothyroglobulin" fraction from 6.97% to 24.7%. The PBI^{127} concentration increased from 2.8 μg.% in the initial sample to 70.0 μg.% in the final sample. The increase in the "radiothyroglobulin" fraction and the marked increase in the PBI^{127} concentration in the circulating medium were attributed to damage of the cells of the thyroid.

The distribution of radioactive compounds in the thyroid, as revealed by column chromatography, was as follows: thyroxine, 2.8%; iodide, 6.5%; diiodotyrosine, 42.8%; monoiodotyrosine, 38.0%; column residual and

TABLE 1. SERIAL CONCENTRATION OF RADIOACTIVITY IN CIRCULATORY COMPARTMENTS DURING PERFUSION OF BEEF THYROID LOBE WITH RADIOIODIDE

Perfusion time, minutes	Arterial concentration, μc. per ml.	Venous concentration, μc. per ml.	Seepage concentration, μc. per ml.	Efficiency,* per cent	T.C.A.-precipitable I^{131},† per cent
15	0.0462	0.0408	0.0467	11.7	3.3
30	0.0404	0.0364	0.0409	9.9	3.5
45	0.0372	0.0324	0.0376	12.9	3.9
60	0.0335	0.0272	0.0332	18.8	4.8
120	0.0217	0.0166	0.0214	23.5	9.3
180	0.0152	0.0131	0.0157	13.8	16.1
240	0.0129	0.0110	0.0133	14.7	21.2
300	0.0114	0.0101	0.0116	11.4	23.9
360	0.0109	0.0096	0.0109	11.9	27.5

* Per cent efficiency $= \frac{Ac - Vc}{Ac} \times 100$. (Ac = Arterial concentration, μc. per ml.; Vc = Venous concentration, μc. per ml.)

† Pooled plasma from arterial, venous and seepage blood was used.

intereffluent, 9.9%. This distribution is similar to that of a normal human thyroid 5½ hours after the administration of I^{131} *in vivo* (Braasch *et al.*, 1955).

In two further experiments with pulsatile perfusion the 6-hour collection of I^{131} by the lobe was 34.8% and 43.8% of the dose. There was incomplete isolation of the venous return in one; therefore venous cannulation and clamping of the tissue were omitted in subsequent perfusions.

2. Gravity Perfusion.—The thyroids were dissected from surrounding tissues and perfused by gravity at a pressure of 50 mm. of mercury. Different mediums were used to determine the effect of each on the thyroidal collection of I^{131}: citrated beef blood, beef serum, and Krebs-Ringer bicarbonate solution containing 0.2% glucose (Umbreit, Burris and Stauffer, 1949) and 6% dextran (Dextran, Pharmacia, Sweden; Hydrodex, Wm. S. Merrell Company, Cincinnati, Ohio). The results of these studies are shown in Table 2.

TABLE 2. UPTAKE, BINDING AND RELEASE OF I^{131} OF BEEF THYROIDS PERFUSED BY GRAVITY WITH DIFFERENT MEDIUMS

Medium	Exp't. No.	Uptake, per cent of dose		Thyroid T.C.A.-precipitable I^{131}, per cent	Slice‡ (4-hr., per cent dose per 100 mg.)	Perfusate T.C.A.-precipitable I^{131}, per cent	
		At 3 hr.	At 6 hr.			Initial	Final
Blood	1	60.4	64.4	85.3	—	3.1	7.0
	2	23.9	28.3	88.6	—	3.7	5.7
Serum	3	33.7	47.9	49.7	—	4.9	7.9
	4	86.2	97.0	95.4	75.0	1.8	23.0
	5	76.8	87.6	95.6	44.6	2.9	14.2
	6	70.8	80.9	94.5	—	3.8	12.3
	7*	23.1	30.8	79.8	—	3.2	4.0
K.R.B.	8	8.8	—	34.1	14.9	—	—
+	9	8.9	—	63.1	20.0	—	—
dextran†	10	43.8	53.4	93.7	50.5	—	—

* Twenty units of thyrotropin was added to the perfusate at 105 minutes.
† Krebs-Ringer bicarbonate solution with 6 per cent dextran.
‡ Slice incubated with I^{131} was taken from contralateral, unperfused lobe (see text).

A wide variability in the collection of I^{131} occurred with each of the three mediums. In five instances, slices from the corresponding unperfused lobe were incubated in Krebs-Ringer bicarbonate solution containing I^{131}. In general, the incubated slices revealed a variability in the collection of I^{131} similar to that of the corresponding perfused lobes, a fact which indicated that a part of the variability was probably inherent in the animals.

Histologic examination of the perfused thyroids revealed focal areas of colloid loss, desquamation and occasionally pyknosis of the follicular cells with each of the mediums used.

The injection of 20 U.S.P. units of thyrotropin (The Armour Laboratories, Chicago, Illinois) 105 minutes after perfusion was started, in Experiment 7, produced no effect on the thyroidal accumulation of I^{131}.

B. Dog Thyroid. 1. Control Group.—Pulsatile pressure was used for the perfusion of both lobes of the thyroid from 12 dogs. The mean curve for

TABLE 3. PERFUSION OF DOG THYROIDS IN THE PRESENCE OF VARIOUS SUBSTANCES AFFECTING THYROID FUNCTIONS

Group	Number	6-hr. uptake, per cent of dose per gm.	Thyroid T.C.A.-precipitable I^{131}, per cent	Perfusate					
				Initial T.C.A.-precipitable I^{131}, per cent	Final T.C.A.-precipitable I^{131}, per cent	Initial		Final	
						I^{127},* μg. per cent	PBI^{127}, μg. per cent	I^{127},* μg. per cent	PBI^{127}, μg. per cent
Control	12	14.1 ±1.59†	78.0 ±3.56	5.7 ±0.40	10.0 ±1.14	3.1 ±0.45	2.5 ±0.26	31.5 ±5.22	25.2 ±5.13
TSH in vivo	2	32.8	81.0	3.3	11.7	3.3	3.5	20.0	20.2
NaI in vitro	1	1.1	25.8	3.8	2.4	—	—	—	—
Thiouracil in vitro	1	13.0	0.7	6.2	6.6	4.4	3.6	17.0	12.0
Thiouracil and KSCN in vitro	2	0.8	1.8	4.3	2.1	14.3	1.9	53.0	31.8
TSH in vitro	2	16.3	79.9	5.7	7.1	1.8	2.8	56.3	52.8

* Total blood I^{127}.
† Standard error of the mean.

the accumulation of I^{131} by the thyroids is shown in Figure 2. The initial slope of the thyroid curve was increased owing to the marked hemorrhage occurring in the extrathyroidal tissue during the first 15 to 30 minutes of perfusion.

The average T.C.A.-precipitable I^{131} in the perfused thyroids was 78% (Table 3) as compared to 98.7% when I^{131} was administered *in vivo* (control, Table 4).

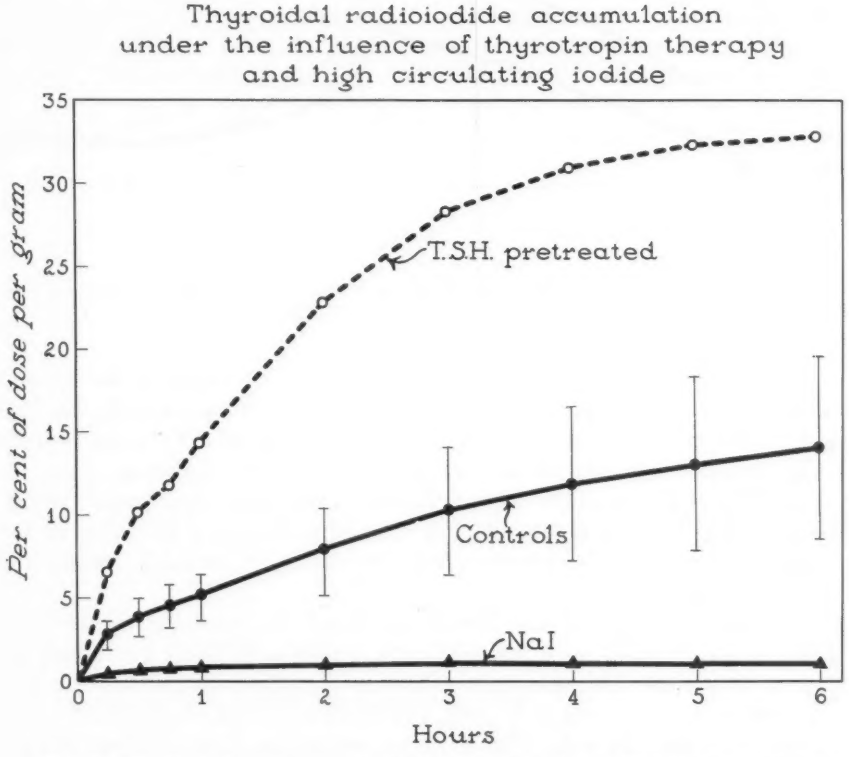

FIG. 2. The effect of pretreatment with thyrotropin and the effect of 10 mg. of sodium iodide in the perfusate on the accumulation of I^{131} by the perfused dog thyroid. The vertical lines on the mean curve of the control group represent ± 1 standard deviation about the mean.

Microscopic examination of the thyroids revealed focal areas of degeneration similar to those occurring with the beef thyroid.

2. Effect of TSH in Vivo.—Two dogs were injected subcutaneously with 10 U.S.P. units of thyrotropin daily for 3 days. Both lobes of the thyroid and blood were removed for perfusion 24 hours after the last injection. The pretreatment with thyrotropin resulted in an increased accumulation of I^{131} by the dog thyroids (Fig. 2 and Table 3).

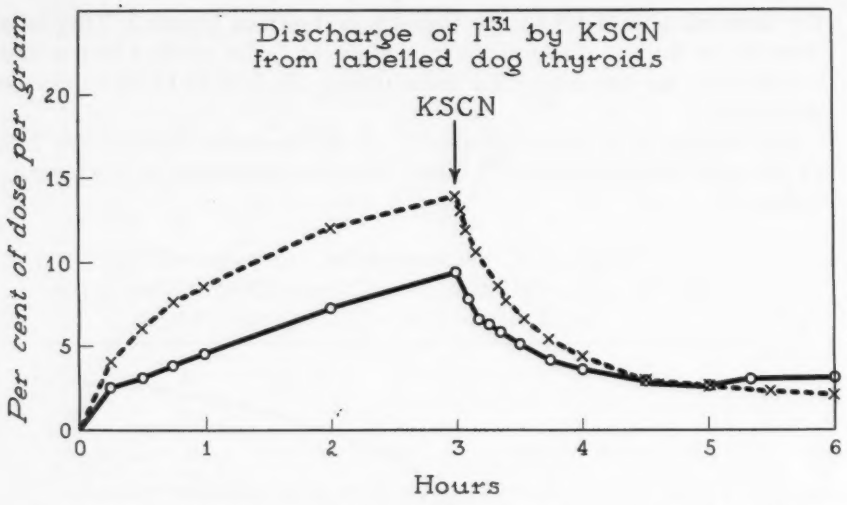

FIG. 3. Discharge of radioactive iodide by potassium thiocyanate from two dog thyroids perfused with heparinized blood containing 0.001 *M* thiouracil.

3. Effect of NaI in Vitro.—In one instance, both lobes of the thyroid were perfused with heparinized blood containing 10 mg. of NaI. This resulted in a marked depression of the accumulation of I^{131} by the thyroid (Fig. 2) and a depression of the organic binding of I^{131} (Table 3).

4. Effect of Thiouracil in Vitro.—In one experiment, the organic synthesis of I^{131} by the thyroid was blocked by adding 26 mg. of thiouracil to the heparinized blood. The 6-hour accumulation of I^{131} was well within the control range (Table 3).

5. Effect of Thiouracil and KSCN in Vitro.—In two experiments, both lobes of the thyroid were perfused with heparinized blood containing 26 mg. of thiouracil. The injection of 50 mg. of potassium thiocyanate into the perfusate, at the end of 3 hours, produced a prompt discharge of radioiodide from the thyroids (Fig. 3). The pattern of discharge was similar

TABLE 4. PERFUSION OF DOG THYROID GLANDS PREVIOUSLY LABELED WITH I^{131}

Group	Thyroid in vivo, per cent of dose per gm.	6-hr. loss, per cent	Thyroid T.C.A.-precipitable I^{131}, per cent	Initial perfusate		Final perfusate	
				I^{127},* μg. per cent	PBI^{127}, μg. per cent	I^{127},* μg. per cent	PBI^{127}, μg. per cent
Control	25.1	2.8	98.7	2.8	1.2	25.0	25.0
TSH†	8.1	4.2	98.5	3.6	1.1	50.0	41.0
TSH and thiouracil†	15.6	9.3	93.7	2.2	0.8	42.0	34.6

* Total blood I^{127}.
† Added to perfusing medium.

to that described in normal and thyrotoxic patients *in vivo* (Stanley and Astwood, 1948).

6. Effect of TSH in Vitro.—In two experiments, both lobes of the thyroid were perfused with heparinized blood containing 20 U.S.P. units of thyrotropin. The results are shown in Table 3. Thyrotropin *in vitro* produced no apparent effect on the accumulation of I^{131}.

7. Effect of TSH on the Labeled Thyroid.—The thyroid glands of three dogs were labeled by administering 50 μc. of I^{131} intravenously 4 days prior to perfusion. In these experiments, the per cent loss of radioactivity from both lobes of the perfused thyroid was determined for 6 hours. Twenty U.S.P. units of thyrotropin were added to the heparinized blood with and

TABLE 5. THE EFFECT OF STORAGE ON THYROID FUNCTION AS DETERMINED BY PERFUSION OF DOG THYROID LOBES

Storage interval	6-hr. per cent of dose per gm.	Thyroid T.C.A.-precipitable I^{131}, per cent	Perfusate			
			Initial		Final	
			I^{127},* μg. per cent	PBI^{127}, μg. per cent	I^{127},* μg. per cent	PBI^{127}, μg. per cent
Control	21.8	86.8	2.2	2.0	25.0	26.2
24 hr.	10.2	71.8	—	—	31.2	28.0
Control	76.8	88.1	1.0	1.0	5.0	7.6
24 hr.	17.5	67.8	—	—	22.0	32.6
Control	41.0	89.4	1.0	1.3	13.0	13.8
48 hr.	10.1	65.3	—	—	248.0	320.0
Control	52.1	94.1	1.6	1.7	11.0	17.6
96 hr.	10.8	81.5	—	—	365.0	495.0
Control	11.8	40.5	5.1	2.1	23.0	16.0
168 hr.†	3.6	32.1	—	—	380.0	580.0
Control	28.4	73.6	2.0	2.4	8.8	9.2
24 hr.‡	1.4	40.7	—	—	1,075.0	1,285.0

* Total blood I^{127}.
† Perfused with blood from a donor dog.
‡ Stored room temperature. Remainder stored 4° C.

without 26 mg. of thiouracil. The maximal 6-hour loss of radioactivity was 9.3% and occurred when thyrotropin and thiouracil were present in the perfusate (Table 4). The magnitude of this loss was not considered great enough to indicate a releasing action of thyrotropin *in vitro*. Wolff (1951) found approximately a 44% loss of radioactivity from the labeled thyroids of rats 7 hours after the administration of thyrotropin and thiouracil.

The I^{127} and PBI^{127} concentrations in the final perfusate of these three experiments were within the range of the previous control group (Tables 3 and 4).

8. Length of Survival Under Different Environmental Conditions.—In 14 perfusions, one lobe of the dog thyroid was perfused 70 to 75 minutes after removal and the other lobe was stored at 4° C. for 24 to 168 hours or at

room temperature for 24 hours and then perfused. The vascular system of the stored lobes was flushed with dog serum before they were stored. The results of these studies are shown in Table 5. The thyroid was still able to collect 3.6% per gram of the dose after 7 days' storage at 4° C. Storage of the thyroid at 4° C. for 24 to 96 hours apparently produced a greater decrease in the collection of I^{131} than in the T.C.A.-precipitable I^{131}. The increased concentrations of PBI^{127} in the perfusate with increased lengths of storage at 4° C. reflected the marked loss of iodine from the perfused thyroid. This loss was due to cellular degeneration and necrosis.

The perfusion of a single lobe of the dog thyroid apparently resulted in a greater accumulation of I^{131} than when both lobes of the thyroid were perfused. The average collection of I^{131} by the control single lobes was 38.7% of the dose per gram (Table 5) as compared to 14.1% of the dose per gram for the control double-lobe perfused thyroids (Table 3).

In one instance, a single lobe of the dog thyroid was perfused for four consecutive 6-hour periods. There was a progressive decrease in the collection of I^{131} with each period and during the last period a loss of I^{131} from the thyroid preparation occurred. Bacterial colonies were observed in a microscopic section of the thyroid.

SUMMARY

Perfusion experiments on beef and dog thyroids indicated that radioiodide was accumulated, synthesized into organic compounds and released in organic form into the perfusate during a period of 6 hours. The collection of I^{131} by the isolated thyroid varied considerably, a variation which was mainly an expression of animal variability. Cellular damage occurred during the 6-hour period of perfusion, as evidenced by histologic examination of the thyroids and by the appearance of thyroglobulin in the perfusate.

Only gross changes in radioiodine metabolism could be detected in the perfusion studies. Sodium iodide inhibited the collection of radioiodide. Thiouracil blocked organic synthesis, and potassium thiocyanate discharged radioiodide from the blocked thyroids. Thyrotropin added to the perfusing medium did not alter the course of radioiodide collection nor did it markedly accelerate the release of labeled organic compounds from previously labeled thyroids.

The storage of a thyroid lobe in the cold caused a diminution in the thyroidal collection of I^{131} but even after 7 days of storage an appreciable accumulation of I^{131} occurred—about one third that of the contralateral unstored lobe. In contrast, a lobe stored at room temperature for 24 hours had an insignificant collection (5% of its control). Storage affected organic synthesis of I^{131} less than total I^{131} accumulation. The concentration of PBI^{127} in the perfusate increased with increased lengths of storage in the cold. This was an index of cellular damage in the thyroid.

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NOTES AND COMMENTS

LOSS OF ADRENAL GLYCOGEN IN THE RAT FOLLOWING STRESS OR TREATMENT WITH VARIOUS HORMONES[1,2]

In previous work on adrenal glycogen, it was shown that the concentration in rats after fasting periods of 8 to 48 hours was almost twice that found in the non-fasted state, and that administration of glucose to fasted animals produced a decrease of the order of 40% in the fasting level of adrenal polysaccharide within 3 hours (Noble and Papageorge, 1955). These observations raised the question as to how the glycogen of adrenal is affected in states recognized as involving increased activity of the adrenal cortex. This paper presents the results of an investigation on the behavior of adrenal glycogen in the 24-hour-fasted rat after subjection to trauma, exposure to cold, or treatment with ACTH, epinephrine, or insulin.

EXPERIMENTAL PROCEDURES

Male rats of the Sprague-Dawley or Holtzman strain, weighing 200 to 300 gm. were used throughout. They had been maintained on a standard commercial chow *ad libitum*, and were housed in a constant temperature room at 24 to 25° C. except during experimental periods of exposure to low temperature. All animals were submitted to a terminal 24-hour fast during which experimental groups were subjected to special treatment. Control groups of comparable body weight were studied concurrently in each series, although neither injection with physiological saline nor administration of water by stomach tube was found to produce a significant alteration in fasting adrenal glycogen concentration in the rat (Noble and Papageorge, 1955).

Tissue removal for analysis was always done in the morning under pentobarbital anesthesia in the following order: left adrenal, right adrenal, liver. Liver glycogen was determined as a test of efficacy of experimental treatment, although no correlation with adrenal glycogen changes was expected or found. Total polysaccharide was determined colorimetrically by use of Dreywood's anthrone reagent (Dreywood, 1946; Morris, 1948; Durham *et al.*, 1950) following customary digestion of tissue with alkali and isolation of glycogen with alcohol essentially according to Good, Kramer and Somogyi (1933). The trichloracetic acid-soluble (TCA-soluble) fraction of adrenal glycogen was also determined in some groups by a modification of the method of Bloom *et al.* (1951). Details of the procedures for total and TCA-soluble glycogen as adapted for adrenal analysis have been described previously (Noble and Papageorge, 1955).

Traumatic treatment entailed removal of the left adrenal 0.5 or 1.5 hours before excision of the right adrenal and the liver. The incision was drawn together and covered with pads during the interval between left and right adrenalectomy, with the rat under anesthesia throughout the whole procedure. The dissected left gland was submitted to analysis without delay, thus providing individual control values for comparison with glycogen concentration of the right adrenal. Exposure to low temperatures was made by placing the rats in a cold room (0.5 to 13° C.) either throughout the whole 24-hour fasting period or for the 3 terminal hours.

ACTH (Armour's ACTHAR), epinephrine (Lederle Laboratories epinephrine hydrochloride, U.S.P.), or insulin (Lilly's Iletin) was injected in a single dose 3 hours before excision of tissues for analysis. In one group of rats, 5 ml. of 30% glucose were given by stomach tube just before injection of insulin. The 3-hour experimental period was employed because it permits manifestation of adrenocortical stimulation as evidenced by reduction in adrenal ascorbic acid and cholesterol (Sayers *et al.*, 1946) or by decreased eosinophile count (Hills *et al.*, 1948), and also allows time for effect of glucose administration on tissue glycogen (Illingworth and Russell, 1951; Noble and Papageorge, 1955). The hormone preparations were diluted just before injection so as to contain the desired dose per 100 gm. of body weight in 0.2 ml

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[2] The data presented in this paper are taken from the dissertation submitted by Nancy Lee Noble in partial fulfillment of the requirements for the degree of Doctor of Philosophy at Emory University, March, 1953.

solution, and were all given intraperitoneally, except that epinephrine was also given subcutaneously. The doses per 100 gm. were: 1 mg. ACTH, 0.02 mg. epinephrine, and 0.2 U. insulin.

RESULTS

1. *Effects of trauma and of exposure to cold on total glycogen of liver and adrenal.*

Table 1 presents the findings after subjection of rats to the stress of trauma or cold. As expected, liver glycogen was not markedly affected, although there was a statistically significant increase in concentration after the half-hour period of laparotomy ($p < 0.02$) and after 24-hour exposure to 0.5° C. ($p < 0.01$). Alteration in adrenal glycogen concentration was usually far more striking, and always involved a decrease from the control fasting level. The fall observed after either period of trauma is significant whether comparison is made with the left adrenal (removed immediately after opening of the ab-

TABLE 1. EFFECTS OF TRAUMA AND OF EXPOSURE TO COLD ON ADRENAL AND LIVER GLYCOGEN IN 24-HOUR-FASTED RATS

	No. of rats	Adrenal glycogen		Liver glycogen
		mg. % ± S.E.[1]	*p*	mg. % ± S.E.[1]
Trauma Experiment (single gland analysis)				
Controls	7			0.15 ± 0.03
Left adrenal		193.6 ± 12.5		
Right adrenal		198.5 ± 13.0		
0.5-hr. Trauma	5			0.36 ± 0.08
Control l.a.[2]		183.4 ± 15.5		
Traum. r.a.[3]		134.3 ± 13.8	<0.05[4]	
1.5-hr. Trauma	5			0.06 ± 0.01
Control l.a.[2]		166.1 ± 10.4		
Traum. r.a.[3]		70.5 ± 8.6	<0.001[4]	
Cold Exposure Experiment (adrenal pair analysis)				
Controls	8	195.4 ± 10.7		0.12 ± 0.02
0.5° C. for 24 hrs.	5	175.8 ± 16.6		0.40 ± 0.08
13° C. for 3 hrs.	4	159.7 ± 10.6	(<0.1)	0.11 ± 0.04
4° C. for 3 hrs.	4	121.5 ± 12.3	<0.01[5]	0.17 ± 0.04
0.5° C. for 3 hrs.	2	62.9		

[1] Standard error (standard deviation of the mean): S.E. = $\sqrt{\frac{\Sigma d^2}{n(n-1)}}$.

[2] l.a. = left adrenal, removed immediately after abdominal incision.

[3] r.a. = right adrenal, removed 0.5 or 1.5 hr. after excision of l.a.

[4] p is for difference from corresponding left adrenal value.

[5] p is for difference from control group of cold-exposure experiment.

domen) or with values found in right gland analysis of untreated controls. However, the 1.5-hour period of injury doubled the decrease found when only 0.5 hour elapsed after beginning the surgical operation.

Exposure to low temperature brought about a significant fall in adrenal glycogen only when imposed for the last 3 hours of the 24-hour fast, and then the decrease became more marked as the intensity of cold was increased (18% loss, not significant statistically at 13° C.; 38% loss at 4° C., and 68% at 0.5° C.). When the rats were kept at 0.5° C. throughout the 24 hours of food deprivation, the glycogen level in the adrenals did not differ significantly from that of animals maintained at 24 to 25° C.

2. *The effects of treatment with ACTH, epinephrine, insulin, and insulin plus glucose on total glycogen of liver and adrenal.*

The data obtained in these experiments (Table 2) demonstrate even more markedly the difference in behavior of glycogen in the two tissues studied. That of liver behaved

as expected: a slight but significant increase after ACTH; a greater increase 3 hours after epinephrine; no effect following insulin treatment unless accompanied by administration of 1.5 gm. of glucose, which resulted in ample replenishment. The glycogen of adrenal, however, had fallen sharply from its normal fasting level 3 hours after treatment with the hormones. The order of decrease was 35 to 40% following injection of 0.1 mg. ACTH or 0.02 mg. epinephrine per 100 gm. rat (the latter either intraperitoneally or subcutaneously). After 0.2 U. of insulin per 100 gm., severe depletion occurred leaving an average of only 31% as much glycogen as found in adrenals of saline-injected controls. However, when 1.5 gm. of glucose were given by stomach tube just before injection of insulin, the concentration was similar to that found in fasted rats given only the glucose, or in non-fasted rats observed in a previous study (Noble and Papageorge, 1955).

3. *The effect of epinephrine and of insulin on the TCA-soluble fraction of adrenal glycogen.*

Six analyses, representing glands from 12 rats, were carried out to determine TCA-extractable glycogen after treatment with epinephrine. The mean value found was 57.5 ± 9 mg. per 100 gm. of fresh adrenal, or $50.4 \pm 5.8\%$ of the total glycogen reported

TABLE 2. EFFECTS OF SINGLE DOSES OF ACTH, EPINEPHRINE, INSULIN, AND INSULIN-PLUS-GLUCOSE ON ADRENAL AND LIVER GLYCOGEN 3 HOURS AFTER ADMINISTRATION TO 21-HOUR-FASTED RATS

Treatment	No. of rats	Adrenal glycogen		Liver glycogen
		mg. % ± S.E.[1]	*p*	mg. % ± S.E.[1]
Saline, i.p. (controls)	8	181.4 ± 5.6		0.07 ± 0.01
1 mg. ACTH/100 gm., i.p.	4	114.7 ± 12.1	<0.001[2]	0.26 ± 0.04
0.02 mg. Epinephrine/100 gm., i.p.	5	117.7 ± 10.5	<0.001[2]	1.23 ± 0.10
0.02 mg. Epinephrine/100 gm., s.c.	6	114.2 ± 6.2	<0.001[2]	1.28 ± 0.09
0.2 U. insulin/100 gm., i.p.	9	56.3 ± 4.2	<0.001[2]	0.08 ± 0.02
5 ml. 30% glucose by mouth and 0.2 U. insulin/100 gm., i.p.	6	99.8 ± 7.6	<0.001[3]	2.12 ± 0.14
5 ml. water by mouth (controls)	7	170.0 ± 15.7		0.11 ± 0.02
5 ml. 30% glucose by mouth	9	108.7 ± 7.5	<0.001[4]	2.93 ± 0.12

[1] Standard error.
[2] *p* is for difference from saline-injected group.
[3] *p* is for difference from group given insulin without glucose.
[4] *p* is for difference from group given water by mouth.

above in similarly treated animals. For the study of insulin effect on the TCA-soluble fraction, 2 or 4 pairs of glands were pooled for each analysis. From the adrenals of 10 rats (3 analyses) the value was 11.4 mg. ± 0.09 mg. of TCA-extractable glycogen per 100 gm. adrenal. This represents only $20.2\% \pm 2.2$ of the total polysaccharide remaining in the adrenal after depletion with insulin as described above.

In the normal, untreated, 24-hour-fasted rat, the adrenal contains 104 ± 7 mg. (9 analyses) of TCA-soluble glycogen per 100 gm., and this is $56.8 \pm 4.2\%$ of its total glycogen concentration (Noble and Papageorge, 1955). Thus 70% of the decrease in total polysaccharide effected by epinephrine (−67 mg.%) was due to loss of the acid-extractable fraction (−46.9 mg.%). The loss in adrenal glycogen after insulin involved 125 mg.% of total glycogen, of which 93 mg. or 74% was TCA-soluble.

In our earlier study it was found that about two-thirds of the difference in adrenal glycogen concentration between fasted and non-fasted rats was accounted for by the acid-soluble fraction. The observations reported in this paper thus support the suggestion previously made (Noble and Papageorge, 1955) that the TCA-extractable fraction of adrenal glycogen is more readily altered than the residual fraction (total glycogen −TCA-soluble), and that the former, therefore, represents the metabolically more labile

moiety of polysaccharide in the adrenal. This agrees with the reported behavior of TCA-extractable glycogen in other tissues of the rat (Bloom, Lewis, Schumpert and Shen, 1951; Bloom and Russell, 1952; Bloom and Knowlton, 1953).

DISCUSSION

The observed behavior of adrenal glycogen bears a strong resemblence to the response in similar circumstances of adrenal ascorbic acid, adrenal cholesterol, circulating lymphocytes and circulating eosinophiles (Long, 1952), suggesting that activation of the adrenal cortex leads to loss of polysaccharide from the gland. Depletion of adrenal glycogen was particularly severe when the stimulus for ACTH release was strong, i.e.: after the longer period of trauma, with greater intensity of cold of 3-hour duration, and after an insulin dose sufficient to cause marked hypoglycemia. The relationship between loss of adrenal ascorbic acid and endogenous or exogenous ACTH has been reviewed by Sayers and Sayers (1949), and there is evidence that cholesterol loss is also increased with greater intensity of stimulus for ACTH release (Sayers, *et al.*, 1944; Botkin and Tew, 1952). Like vitamin C, glycogen falls sooner than cholesterol and returns to the normal fasting level by the end of 24 hours of cold exposure, whereas cholesterol is still depressed at this time (Long, 1947). However, glycogen loss from the adrenal appears to be manifested at a lower level of adrenocortical activity than that required for ascorbic acid disappearance.

Adrenal ascorbic acid is not appreciably affected by normal ingestion of food or by oral or parenteral glucose (Dury, 1950; Vogt, 1951; Noble and Papageorge, unpublished data), and loss induced by insulin can be prevented if hypoglycemia is forestalled by glucose administration (Gershberg and Long, 1948; Vogt, 1951). Adrenal glycogen, however, remained significantly below the normal fasting level when an oral dose of 1.5 gm. of glucose accompanied insulin injection, although the depletion was less severe than when insulin alone was given. Chamberlain (1930) reported similar findings with adrenal cholesterol in rabbits when insulin hypoglycemia was prevented by glucose. Lymphocytopenia and eosinopenia occur within 1 to 3 hours after carbohydrate administration to overnight-fasted rats (Dury, 1950; Elmadjian, *et al.*, 1946; Steeples and Jensen, 1949), to fasted dogs (Jordan, *et al.*, 1950), and to human subjects (Pincus, *et al.*, 1949). Appreciable decrease in adrenal cholesterol follows forced feeding of 1 gm. of carbohydrate per 100 gm. to fasted rats, but the loss is not evident until 7 to 8 hours after ingestion of the sugar (Abelin, 1945; Steeples and Jensen, 1949). Further arguments supporting the view that administration of glucose leads within a few hours to appreciable release of cortical hormones in the intact animal have been given by Constantinides, Fortier and Skelton (1950).

One may suggest that the concentration of adrenal glycogen after relatively short periods of fast represents a basal state in which the pituitary-adrenal system of the rat is quiescent. Any disturbance which provokes release of ACTH and, therefore, leads to stimulation of the adrenal cortex may thus result in reactions in the adrenal which are reflected by loss of glycogen. Since the role of glycogen in adrenal metabolism is even more obscure than that of either ascorbic acid or cholesterol, one can only speculate as to why polysaccharide disappears from the activated gland. Is the glycogen metabolized along with other substances which supply energy to meet increased needs following stimulation, or does oxidation of glycogen represent a purposeful reaction for procurement of hydrogen in the synthesis of cortical steroids? At the present time, such speculation is of little value but this investigation suggests that further study of the relation between activity and glycogen concentration of the adrenal may lead to interesting findings.

SUMMARY

Subjection of 24-hour-fasted rats to the trauma of laparotomy for 0.5 to 1.5 hours or to an environmental temperature of 0.5 to 4° C. for the last 3 hours of the fast resulted in significant loss of adrenal glycogen. However, when exposure to cold was maintained throughout the 24 hours of fasting, adrenal glycogen concentration did not differ significantly from the control value of 195 mg.%.

Injection of ACTH, epinephrine or insulin in respective doses of 1 mg., 0.02 mg. and 0.2 U. per 100 gm. of body weight decreased adrenal glycogen concentration significantly within 3 hours in the 24-hour-fasted rat. The order of decrease after ACTH or epinephrine was 40%, and after insulin 70%. If, however, 1.5 gm. of glucose were given orally just before insulin injection, adrenal glycogen loss 3 hours later was only 45%, the same as that found when such a dose of glucose was given without insulin to fasted animals.

Depletion of the trichloracetic acid-soluble fraction accounts for about 70% of the loss of total adrenal glycogen effected by treatment with epinephrine or insulin.

It is suggested that reduction in glycogen concentration of the gland reflects stimulation of the adrenal cortex.

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DEPLETION OF ADRENAL ASCORBIC ACID FOLLOWING STRESS IN THE INFANT RAT[1]

In an earlier note the presence of ACTH in the pituitary gland of the infant rat, 4–7 days old, was reported (Rinfret and Hane, 1955a). Administration of acid extracts of the infant pituitary gland produced depletion of adrenal ascorbic acid not only in more mature hypophysectomized rats, but in infant rats of the same age group as the pituitary donors. Since the endocrine components believed necessary to produce a stress response (i.e. depletion of adrenal ascorbic acid) were demonstrably present in these very young animals, it was decided to test their capacity to respond to severe stress. It is the purpose of this communication to report the depletion of adrenal ascorbic acid in infant rats, 4–7 days old, following laparotomy.

MATERIALS AND METHODS

Infant rats of the Wistar strain, 4–7 days of age, were subjected to laparotomy. Following 0–6 hours in this state, the animals were sacrificed. Two modifications in procedure were used. In seven experiments the left adrenal were removed at the start of the experiment and the glands from 3 to 5 animals were pooled to make a single ascorbic acid determination. At the end of the test period the right adrenals from the same animals were pooled. In four experiments the right and left adrenal glands from 3 animals were combined following the stress period. The ascorbic acid values obtained provided a single observation which was compared to that for unstressed controls. In all tests control animals were sacrificed during the experimental period.

Immediately after trimming adhering connective tissue, the glands were weighed and frozen in solid carbon dioxide. The concentration of adrenal ascorbic acid was determined by the method of Roe and Kuether (1943).

RESULTS

In 10 of 11 experiments a significant depletion of adrenal ascorbic acid (p .05 or less) was observed in the 4–7 day old rats following laparotomy. The results are summarized in Tables 1 and 2.

DISCUSSION

The pituitary-adrenal response of infant rats to the severe stress of laparotomy is qualitatively the same as that observed in more mature animals, insofar as this response is susceptible to investigation by the determination of adrenal ascorbic acid. The hypophysis of the very young animal contains ACTH, and its adrenal is responsive to that material when exogenously administered. The finding of a measurable response to severe stress implies that the excitatory apparatus involved is present and functional in the rat 4–7 days old.

Certain quantitative differences between the infant and more mature animal are apparent. The concentration of adrenal ascorbic acid in the former is about 60% of the

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TABLE 1. ADRENAL ASCORBIC ACID DEPLETION IN INFANT RATS FOLLOWING STRESS (LAPAROTOMY)

Exp't. No.	Group	Duration of stress	Number of observations	Number of animals	Mean adrenal AA* depletion, mg. %, St. Dev.	P
1	(a)	1 hour	4	(15)	0 ± 36	
	(b)	2 hours	4	(16)	35 ± 39	< .5, > .1
	(c)	3 hours	5	(20)	52 ± 30	.05
2	(a)	None	3	(12)	24 ± 55	
	(b)	3 hours	7	(30)	94 ± 34	< .05, > .02
3	(a)	None	4	(16)	13 ± 5	
	(b)	3 hours	10	(42)	75 ± 22	< .001
4	(a)	0–1 hour	8	(31)	1 ± 23	
	(b)	2–6 hours	16	(63)	34 ± 22	< .05, > .02
5	(a)	0–1 hour	10	(43)	1 ± 23	
	(b)	2–6 hours	17	(73)	22 ± 24	< .05, > .02
6	(a)	None	5	(19)	2 ± 14	
	(b)	3–6 hours	19	(77)	35 ± 38	< .10, > .05
7	(a)	None	7	(29)	−7 ± 19	
	(b)	3–6 hours	18	(71)	29 ± 32	< .01

* Value obtained by subtracting concentration of ascorbic acid in right adrenals from that in left. Thirty-one observations on a total of 125 animals, sacrificed without stress, yielded a mean difference in concentration of ascorbic acid between left and right adrenals of 5 mg. % ± 24.

latter. The severe stress of laparotomy appears to require at least 2 hours to produce an unequivocal response in a group of infant rats, while the same treatment in more mature animals results in a sharp depletion of adrenal ascorbic acid in one hour (Rinfret, 1955b).

TABLE 2. ADRENAL ASCORBIC ACID CONCENTRATIONS IN STRESSED AND UNSTRESSED INFANT RATS

Exp't. No.	Group	Duration of stress	Number of observations	Number of animals	Mean adrenal AA concentration mg. %, Std. Dev.	P (control)
8	(a)	None	8	(24)	260 ± 37	
	(b)	2 hours	8	(24)	242 ± 39	> .10
	(c)	4 hours	9	(27)	212 ± 34	< .02, > .01
	(d)	6 hours	8	(23)	201 ± 31	< .01
9	(a)	None	9	(27)	231 ± 42	
	(b)	2 hours	8	(24)	177 ± 22	< .001
	(c)	4 hours	8	(24)	180 ± 25	< .01
	(d)	6 hours	8	(25)	186 ± 33	< .01
10	(a)	None	9	(28)	229 ± 19	
	(b)	2 hours	8	(24)	208 ± 19	< .02, > .01
	(c)	4 hours	8	(24)	195 ± 25	< .01
	(d)	6 hours	8	(25)	204 ± 22	< .01
11	(a)	None	8	(24)	231 ± 10	
	(b)	2 hours	8	(24)	178 ± 11	< .001
	(c)	4 hours	8	(24)	198 ± 18	< .001
	(d)	6 hours	9	(27)	192 ± 8	< .001

This observation and the reports of Jailer (1950) and Irwin *et al.* (1950) suggest that the pituitary-adrenal axis of the newborn is somewhat less sensitive to a potentially stressful situation than that of the older animal. This difference in sensitivity cannot be attributed to a deficiency of pituitary ACTH nor to an inability of the adrenal to deplete its stores of ascorbic acid, nor to the absence of a functional excitatory mechanism which may participate in the response to the stress used.

SUMMARY

A pituitary-adrenal response to stress (laparotomy) in the infant rat, 4–7 days old, is qualitatively the same as that seen in more mature animals. Depletion of adrenal ascorbic acid was observed at 2 through 6 hours following initiation of stress.

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DETECTION OF C-21 GLYCEROL SIDE CHAIN STEROIDS ON PAPER CHROMATOGRAMS

Metabolic studies of the adrenocorticoids emphasize the need for a simple sensitive and specific method for the detection on paper partition chromatograms of steroids with a 17, 20, 21 trihydroxy side chain. The method to be described depends on the oxidative removal of the glycerol side chain with the production of a 17-ketosteroid which may then be detected by the Zimmermann reaction for these compounds.

MATERIALS AND METHODS

Reagents

a. Oxidizing reagent: A 1% solution of either sodium metaperiodate or of periodic acid in 10% aqueous tetramethyl ammonium hydroxide. This solution is quite stable.

b. Zimmermann reagent: A 2% solution of purified dinitrobenzene in absolute ethanol. A 2.5 N aqueous or ethanolic solution of potassium hydroxide.

Oxidation step: Draw a test strip cut from the paper chromatogram through the oxidizing reagent quickly. This is important if a fragile paper such as Whatman #1 or #42 is used. Blot the test strip between filter paper and hang up to dry in the darkness for three to twenty-four hours at room temperature. It is not necessary to remove excess oxidizing reagent.

To detect the 17-ketosteroid formed a Zimmermann test is performed on the test strip. Draw the strip through the KOH solution, blot it between filter paper, and then pull it through the dinitrobenzene solution. Dry the strip in an oven at 60° C. for about 1½ minutes. If color development is doubtful return the strip to the oven for another ½ to ¾ of a minute longer. This Zimmermann technique is suggested: other standard techniques for use on paper have been tried and may equally well be substituted.

Unless it is known that there is no Zimmermann reactive material present before the oxidative step a simultaneous control Zimmermann test must be done on an unoxidized test strip. The color of the oxidized strip is then compared with that of the control strip to differentiate the colors produced by a 17-ketosteroid and a Δ^4-3-ketosteroid or a 3-ketosteroid. Table 1 lists compounds tested.

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TABLE 1. COMPOUNDS TESTED BY THE OXIDIZED ZIMMERMANN TEST

Compound	Side chain	Amount micrograms per cm.²	Zimmermann reaction	
			Oxidized strip	Control strip
Allopregnane-3β, 11β, 17α, 20β, 21-Pentol (Reichstein's Substance "A")	CH_2OH | CHOH | ···OH	50 40 20 10 5	Strong positive Strong positive Strong positive Positive Faintly positive	Negative Negative Negative Negative Negative
Δ^4-Pregnane-17α, 20β, 21-triol-3-one	CH_2OH | CHOH | ···OH	100 50 25 10 5	Strong positive Strong positive Strong positive Positive Slightly positive	Positive Slightly positive Doubtful Doubtful Negative
Δ^4-Pregnane-17α, 20α, 21-triol-3-one	CH_2OH | CHOH | ···OH	100 50 25 10 5	Strong positive Strong positive Strong pisitive Positive Slightly positive	Faint Very faint Doubtful Negative Negative
Δ^4-Pregnene-21, 20α-diol-3-one	CH_2OH | CHOH	100 20	Positive Slightly positive	Slightly positive Faint
Δ^4-Pregnene-21, 20β-diol-3-one	CH_2OH | CHOH	120 25	Positive Slightly positive	Positive Faint
Pregnane-3α, 17α, 20α-triol	CH_3 | CHOH | ···OH	100 50	Negative Negative	Negative Negative
170H Progesterone	CH_3 | C=O | ···OH	50	Negative	Negative
Allopregnan-3α, 20α-diol	CH_3 | CHOH	50	Negative	Negative
Progesterone	CH_3 | C=O	50	Faint	Faint
Pregnane-3α, 11β, 17α, 21-tetrol-20-one (Tetra F)	CH_2OH | C=O | ···OH	50	Negative	Negative
Δ^4-Pregnene-11β, 17α, 21-triol-3,20-dione (Hydrocortisone) Cpd. F	CH_2OH | C=O | ···OH	50	Faint	Faint
Δ^4-Pregnene-17α, 21-diol-3,11, 20-trione (Cpd. E)	CH_2OH | C=O | ···OH	50	Very faint	Faint

TABLE 1—(*continued*)

Compound	Side chain	Amount micrograms per cm.²	Zimmermann reaction	
			Oxidized strip	Control strip
Corticosterone Δ^4-Pregnene, 11β, 21-diol,3,20-dione	CH_2OH | $C=O$	50	Faint	Faint
Desoxycorticosterone Δ^4-Pregnene-21-ol-3,20-dione	CH_2OH | $C=O$	50	Negative	Negative
Δ^4-3-ketoetienic acid		100 20	Positive Faint	Slightly positive Negative
Cholesterol purified through the dibromide		100 50	Negative Negative	Negative Negative

DISCUSSION

Periodic acid oxidation of steroids is frequently carried out to obtain structural information (Jackson, 1944) and for quantitative analytical purposes (Daughaday *et al.*, 1948). In these applications oxidation takes place in an acid or a neutral medium where the oxidation potential is high (Syrokaniskii and Melamed, 1950) and the oxidation rate is rapid (Fleury *et al.*, 1952; Price and Kroll, 1938). Under these conditions 20, 21 α-ketols yield formaldehyde and etianic acids; 20, 21 glycols yield compounds having a formyl group at C-17; and 17, 20 dihydroxysteroids form 17-ketosteroids (Jackson, 1944). The extent of the reaction is quantitative or nearly so in most instances. We were not able, however, to obtain satisfactory Zimmermann tests on paper when the oxidation step was carried out under aqueous acid conditions.

In alkaline medium the oxidation potential of periodate is reduced (Syrokaniskii and Melamed, 1950) and this is also true for the oxidation rate for glycols (Prince and Kroll, 1938). Under conditions of the test described in the experimental part only those steroids with a glycerol side chain at C-17 were found to yield a strong Zimmermann test for 17-ketosteroids; 17, 20 diols were negative and only weak Zimmermann tests were obtained with 21, 20α and 21, 20β dihydroxydesoxycorticosterones.

Under conditions of Bush (1955) where oxidation is conducted in a strongly alcoholic and acid medium the rate of reaction is also reduced (Fleury *et al.*, 1952) but 17, 20 diols still react and thus specificity is reduced. We have used this test for several months in the routine examination of paper chromatograms.

Because of interference with the Zimmermann color in solution by the oxidation reagents the method as described is not readily adapted for quantitative estimations.

SUMMARY

The alkaline periodate oxidation of steroids with a 17, 20, 21 trihydroxy (glycerol) side chain results in the formation of a 17-ketosteroid which may be specifically detected on paper chromatograms by the Zimmermann test.

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IODIDE AND THE THYROIDAL MORPHOLOGIC RESPONSE TO EXOGENOUS THYROTROPHIN IN HYPOPHYSECTOMIZED RATS[1]

Rawson (1949) has reported that large doses of iodide almost completely inhibit the increase in thyroid cell height which is obtained when hypophysectomized rats are treated with thyrotrophin (TSH). The present study was undertaken to determine whether such an interference with the morphologic effects of TSH is due to a) high levels of serum iodide *per se*, b) high levels of "trapped" iodide within the thyroid, c) an increase in the thyroidal concentration of compounds which contain iodine in organically bound form, or d) enhanced titers of circulating thyroid hormone. However, the only impressive result of this investigation was our inability to alter profoundly the thyroidal morphologic response to TSH by means of iodide.

MATERIAL AND METHODS

All animals used in this study were hypophysectomized male rats (150–200 gm. body weight) purchased from the Hormone Assay Laboratories, Chicago, and maintained on a Remington diet.[2] Thyrotrophin (TSH)[3] was dissolved in saline and injected subcutaneously. When propylthiouracil (PTU) was given, it was administered by the same route (as a suspension) in daily doses of 10 mg. Iodide was either injected subcutaneously in two daily doses of 500 μg. of NaI each, or was mixed with the diet in concentrations of 1.8 or 18 mg. of iodide per kg. of feed. When KSCN was given, it was mixed into the feed at a concentration of 0.5%. Most animals were started on the regimens 7 days postoperatively. The rats in experiments I and IV were given their first injection on the fifth day after hypophysectomy. TSH and PTU were last administered on the day before the sacrifice. When iodide was injected, the animals received 500 μg. of NaI on the day of the sacrifice; the feeding of the regimens fortified with iodide or containing KSCN was continued until 2 hours before the death of the animals. Thyroid cell height measurements and, in some groups, thyroid:serum iodide gradient determinations were carried out as described in earlier studies (Halmi *et al.*, 1953, Halmi, 1954).

RESULTS AND DISCUSSION

The results of the study are summarized in Table 1. Experiments I and IV, II and V, and III and VII were carried out simultaneously. The data of Experiments I and IV

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[2] Low Iodine Test Diet, Nutritional Biochemicals, Inc., Cleveland, Ohio.
[3] USP Thyrotropin Reference Substance (Experiments I and IV) and Armour Lot #P589-80 (all other experiments). The latter was generously furnished by Dr. S. L. Steelman.

have been previously published (Halmi and Spirtos, 1955) and are included in this report to facilitate comparison with subsequently obtained findings.

All TSH-treated rats, whether they received iodide treatment or not, showed thyroid cell heights which were significantly ($P < 0.01$) above those of untreated hypophysectomized rats sacrificed 15 days postoperatively (controls of Table 1).

Organic binding of iodine by the thyroid was not blocked in Experiments I to III. No depression of the cell height response to TSH was observed when the basic diet was fortified with 1.8 or 18 mg. of iodide per kg. (Experiment I), although the efficiency of the thyroidal "trapping" of iodide was markedly dependent on and inversely proportion-

TABLE 1. ANALYSIS OF THE EFFECT OF IODIDE ON THE THYROIDAL CELL HEIGHTH RESPONSE TO THYROTROPIN (TSH) IN HYPOPHYSECTOMIZED RATS MAINTAINED ON A REMINGTON DIET

Exper. No.	Duration of exper., days	No. rats	TSH USP u./day	PTU mg./day	Iodide supplement	KSCN in diet	Thyroid cell height, μ	
Control		17	—	—	—	—	4.7 ± .10*	
I	10	10	1.0	—	—	—	9.9 ± .25	
	10	11	1.0	—	1.8 mg./kg. diet	—	10.9 ± .29	
	10	10	1.0	—	18 mg./kg. diet	—	10.8 ± .39	
II	7	10	0.18	—	—	—	7.6 ± .31	
	7	10	0.18	—	1 mg. NaI/day	—	7.3 ± .31	
III	7	16	0.9	—	—	—	11.8 ± .23	$p < 0.01$
	7	14	0.9	—	1 mg. NaI/day	—	9.7 ± .36	
IV	10	18	1.0	10	—	—	11.5 ± .30	$p < 0.01$
	10	14	1.0	10	1.8 mg./kg. diet	—	10.3 ± .37	
	10	16	1.0	10	18 mg./kg. diet	—	9.6 ± .36	
V	7	5	0.18	10	—	—	7.7 ± .12	$p < 0.01$
	7	6	0.18	10	1 mg. NaI/day	—	6.5 ± .29	
VI	7	18	0.18	10	—	—	9.4 ± .19	$p < 0.01$
	7	13	0.18	10	1 mg. NaI/day	—	8.4 ± .12	
	7	12	0.18	10	1 mg. NaI/day	0.5%	8.4 ± .17	
VII	7	12	0.9	10	—	—	10.1 ± .34	
	7	8	0.9	10	1 mg. NaI/day	—	9.9 ± .33	

* Mean ± standard error.

ate to the iodine intake in the same animals (Halmi and Spirtos, 1955). Even the definitely pharmacologic dose of 1 mg. of NaI daily was unable to influence the increase in thyroid cell height elicited by 0.18 USP unit of TSH per day (Experiment II). When 0.9 USP unit of TSH daily was given (Experiment III), there was a significantly smaller but nevertheless very substantial cell height increment in the rats which were also injected with iodide. The reason for the apparent slight inhibition of the thyroidal morphologic response in this particular group is not clear. It is possible that the treatment with large doses of both iodide and TSH resulted in blood thyroxine levels sufficiently high to impede the effect of TSH on thyroid cell growth. Such an inhibition of TSH action has been observed by Cortell and Rawson (1944) in hypophysectomized rats treated with TSH and thyroxine. We are inclined to believe with D'Angelo (1955) that this phenomenon is not due to an interaction of thyroxine and TSH in the thyroid, but rather to an enhanced rate of TSH destruction or elimination under the influence of high titers of thyroid hormone. In the iodide-treated groups of the experiments thus far discussed the concentration of hormonal iodine within the thyroid was presumably higher than in

the rats whose iodine intake was low (Taurog and Chaikoff, 1946). The evidence on hand does not suggest that such an elevation in thyroidal hormonal iodine stores diminishes the morphologic responsiveness of the follicular cells to TSH, although it evidently interferes with the activation of the iodide "trap" (Halmi and Spirtos, 1955).

In Experiments IV to VII organic binding of iodine in the thyroid was blocked with propylthiouracil (PTU). The thyroidal "trapped" iodide concentration in these rats must have been considerably higher than in comparable rats in which iodine binding was permitted to proceed, since a) blocking of iodine-organification by PTU *per se* increases the level of iodide within the thyroid (Wollman and Scow, 1953, 1955) and b) TSH is more effective in promoting "trapping" when it is given in conjunction with PTU (Halmi *et al.*, 1953). As it was furthermore shown in the rats of Experiment IV (*vide* Halmi and Spirtos, 1955) that enhanced iodine intake does not depress the iodide "trap" when PTU is given, the administration of large amounts of iodide to hypophysectomized rats treated with TSH and PTU must have resulted in a tremendous rise of the intrathyroidal "trapped" iodide titers. On the other hand, the serum iodide concentrations in the rats of Experiments IV to VII were presumably of the same order of magnitude as in the comparable groups of Experiments I to III. It therefore appears logical to ascribe the statistically significant depression of the cell height response to TSH that was observed in the high iodine groups of Experiment IV and V, but not in the parallel groups of Experiment I and II, to an elevated level of "trapped" iodide in the blocked thyroids rather than to a rise in serum iodide titers *per se*. Attempts to corroborate this thesis by means of administering KSCN along with iodide (Experiment VI) were unsuccessful. Although KSCN did depress the thyroid:serum iodide gradient (Experiment VI, group 2: 75.3 ± 3.9[4], group 3: 18.8 ± 2.1), this effect was not sufficiently great to eliminate the influx of a plethora of iodide into the thyroid, and thyroid cell heights remained depressed as compared with those of rats on the low iodine regimen (Experiment VI, group 1). It must be emphasized that the iodide-induced depression of the thyroid cell height response to TSH in Experiments IV to VI was not very substantial. In Experiment VII iodide administration altogether failed to influence the follicular cell height increase elicited by TSH.

In conclusion, Rawson's (1949) concept that "the reaction between thyrotrophic hormone and thyroid tissue can be prevented by iodide not only *in vitro* but also in the hypophysectomized rat" is of questionable validity. Iodide as such does not modify the effect of TSH on the thyroidal iodide concentrating mechanism (Halmi and Spirtos, 1955). Wolff (1951) was unable to diminish the enhanced release of hormonal iodine from the thyroid which was due to TSH by concurrently administered large doses of iodide. The present study indicates that high thyroidal "trapped" iodide levels may temper the cell height response to TSH, but that this effect is by no means dramatic or even consistently obtainable.

SUMMARY

High levels of "trapped" iodide within the thyroid may slightly diminish the increase in thyroid cell height which exogenous TSH elicits in hypophysectomized rats. However, iodide administration was not found to exert a profound effect on the morphologic response of the thyroid to TSH.

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[4] Mean ± standard error.

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EFFECT OF HYPOPHYSECTOMY, THYROIDECTOMY, ADRENALECTOMY AND OVARIECTOMY ON ACETATE INCORPORATION INTO LIVER AND CARCASS FATTY ACIDS IN RATS *IN VIVO*

Very little data have been published on the effect of hypophysectomy, adrenalectomy, thyroidectomy or ovariectomy on lipogenesis *in vivo*. Welt and Wilhelmi (1950), using deuterium as a label, found that adrenalectomy increased fatty acid synthesis in the extra-hepatic tissues. While more information can be found on the effect of various hormonal treatments on lipogenesis in liver slices (Brady, Lukens, and Gurin, 1951; Spirites, Medes, and Weinhouse, 1953), the interpretation of such results in terms of the the intact animal is difficult, if not impossible. Therefore, it appeared useful to see how removal of endocrines affected the incorporation of C^{14}-carboxyl labeled acetate into carcass and liver fatty acids. A "short term" technique of acetate incorporation has already been applied to different types of experimental obesity (Bates, Zomzely and Mayer, 1955; Mayer and Zighera, 1955). Because of the possibility that adult rats would be affected differently from growing rats both types of animals were studied.

MATERIALS AND METHOD

Animals used throughout the experiments were female rats of the Sprague-Dawley strain. The operated animals were obtained commercially. The diet consisted of Purina Dog Chow of the following approximate composition; crude protein (min.) 23.1%, crude fat (min.) 5.0%, crude fiber (max.) 6.0%, nitrogen free extract (min.) 44.0%.

The young animals weighed, on the average, 50–100 gm. They were kept in stock cages until they were used. This period was 1–2 weeks from the time of the operation. Water and food were available at all times; the adrenalectomized rats were maintained on physiological saline in place of drinking water. The rats were taken from the cages and given intraperitoneal injections of C^{14}-carboxyl labelled sodium acetate. One-half milliliter containing .362–.728 mg. of acetate was given to each animal. One milligram of the acetate contained 1.9×10^6 cpm. One-half hour after the injection the animals were killed by overetherization and the livers removed. The livers and carcasses were saponified separately in alcoholic KOH solution, the solutions were acidified and the fats extracted with petroleum ether. The fats were plated directly on a planchet lined with lens paper. The carcass samples were done in duplicate. The counts reported are total counts and are expressed as the percent of the injected counts multiplied by a factor of 10^3. The method of fat extraction is described in more detail in a previous paper (Bates, Zomzely and Mayer, 1955).

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C^{14} INCORPORATION INTO CARCASS AND LIVER FATTY ACIDS OF GROWING AND ADULT HYPOPHYSECTOMIZED, ADRENALECTOMIZED, THYROIDECTOMIZED AND OVARIECTOMIZED RATS ONE-HALF HOUR AFTER AN INTRAPERITONEAL INJECTION OF RADIOACTIVE ACETATE

Type of animal	No. of animals	Body weight	Carcass			Liver			Total	
			Wt., gm.	Per cent fat	Counts retained	Wt., gm.	Per cent fat	Counts retained	Total counts retained	Per cent liver synthesis
Control										
Growing	25	76 ± 19	72 ± 18	5.26 ± 1.53	321 ± 87	4.01 ± 1.10	2.79 ± .26	47 ± 23	367 ± 101	13 ± 5
Adult	7	225 ± 10	215 ± 19	9.3 ± 3.3	257 ± 31	9.78 ± .77	3.01 ± .31	56 ± 23	333 ± 33	17 ± 5
Hypophysectomized										
Growing	10	54 ± 8	51 ± 7	4.70 ± 1.41	306 ± 113	2.83 ± .65†	2.48 ± .22†	13 ± 7†	319 ± 117	4 ± 2†
Adult	6	184 ± 8	177 ± 8	6.6 ± 2.9	314 ± 77	7.01 ± .48†	2.79 ± .22	11 ± 10†	325 ± 82	3 ± 2†
Adrenalectomized										
Growing	13	77 ± 18	73 ± 16	4.04 ± 1.64*	329 ± 76	3.65 ± 1.34	2.58 ± .47*	36 ± 23	365 ± 97	9 ± 4†
Adult	6	221 ± 10	212 ± 9	4.1 ± 1.0†	338 ± 91	9.16 ± .76	2.82 ± .29	36 ± 13	374 ± 100	10 ± 2*
Thyroidectomized										
Growing	9	79 ± 10	75 ± 9	4.75 ± 1.16	258 ± 65*	3.79 ± .56	2.79 ± .27	21 ± 8†	279 ± 70*	8 ± 2†
Adult	5	210 ± 10	201 ± 10	5.2 ± .6*	244 ± 38	9.26 ± .89	2.90 ± .22	28 ± 13	272 ± 48*	10 ± 4
Ovariectomized										
Growing	13	96 ± 16	91 ± 15	5.02 ± .59	314 ± 105	5.14 ± 1.26	2.66 ± .14	39 ± 20	331 ± 83	12 ± 5
Adult	7	228 ± 12	218 ± 11	8.7 ± 2.5	283 ± 79	10.14 ± 1.00	3.04 ± .19	49 ± 8	332 ± 78	16 ± 3

* Difference between experimental group and control group significant at 5% level.
† Difference between experimental group and control group significant at 1% level.
Numbers after ± standard deviation.

The adult female rats weighed 175–240 gms. They were put in single cages so that rate of weight gain and food intake could be measured for each rat for a period of a week. In each group animals with similar food intakes were selected for measurement of C^{14} incorporation. The animals were injected with 0.5 ml. of a solution containing 2.66–2.80 mg. of C^{14}-carboxyl labelled acetate solution. One milligram of the acetate contained on the average 1.9×10^6 cpm. The animals were killed as before and the total count and total fat determined.

RESULTS AND DISCUSSION

The authors acknowledge at the outset the limitations of the short term acetate incorporation method as used here. First, it is difficult to eliminate the possibility that differences in diurnal patterns of food intake and of abdominal blood flow between controls and treated animals may not compromise the validity of a "spot check" of lipogenesis from acetate. Perhaps more critical still, as regards interpretation of data is the fact that lipogenesis from acetate may not be indicative of lipogenesis as a whole. Lipogenesis from carbohydrates, for instance, involves a number of stages, and a number of possibilities of control prior to the acetate (or acetyl Co-A) stage. Finally, only adequate determinations of the "2-C fragment flow" or more loosely, "acetate pool," now under study, will permit to gauge the possible effects of dilution on apparent rates of acetate incorporation. On the other hand, the short term acetate method allows to differentiate between carcass and liver lipogenesis *in vivo*, which the deuterium uptake method as used by Welt and Wilhelmi (1950), while more physiological in many respects, does not because it requires eight days for accurate determinations of uptake. Carcass and liver retention of C^{14} counts are shown in the table.

During the seven days that food intake and weight gain were determined on the adult rats, the adrenalectomized rats ate less than the controls though their weight gains were similar. The thyroidectomized rats ate more and gained more weight. The values were obtained over a short period of time and are probably representative of the recovery phase after the operation, rather than of hormonal insufficiency. Hypophysectomy markedly decreased acetate incorporation into liver fat both in the growing and adult animals. Adrenalectomy had no significant effect on either carcass or liver incorporation. In the adult animal thyroidectomy decreased acetate incorporation into fatty acids (though the decreases in carcass and liver fatty acids separately were not significant). In the younger animals thyroidectomy depressed both carcass and liver synthesis of fatty acids. Ovariectomy had no effect on acetate incorporation in either age group. Hypophysectomy, adrenalectomy and thyroidectomy depressed liver synthesis to a greater extent than carcass synthesis, resulting in a lower proportion of liver synthesis to total synthesis. The adrenalectomized and growing thyroidectomized rats contained less fat than their controls.

The increase in lipogenesis in the carcass of the adrenalectomized adult rat failed to be significant at the 5% level. Welt and Wilhelmi (1950) reported an increased deuterium incorporation in carcass lipids. These investigators also found an increased lipogenesis in the liver although the difference failed to be significant at the 5% level.

The adrenalectomized rats had a significantly lower fat content than the controls. This has been described before and has been ascribed to reduced intake of food (Welt and Wilhelmi, 1950; Stoerk and Porter, 1950). Though the animals used here gained as much weight as the controls during the experimental period it is possible that fat loss may have occurred before the animals were put under observation.

The thyroidectomized rats, especially the younger animals show an apparent decrease in acetate incorporation into fat even in spite of the large food intake. Conflicting evidence as to the role of the thyroid in lipogenesis has been published. MacKay and Sherill (1941) found that thyroidectomized animals had a lower fat content than controls. Their "plump" appearance was ascribed to myxedematous changes. Bratzler, Barnes and Swift (1949) reported that when thiouracil was added to the diet there was

an increase in the fat content of the animals. Kennelly and Maynard (1953) reported similar results for young rats receiving thiouracil in the diet.

No difference was found in the acetate incorporation into carcass and liver lipids of ovariectomized rats and control rats in spite of the superior weight gain of comparable ovariectomized rats. This increased weight seems to be due to general increases in size rather than to an increase in fat content (Holt, Keeton, Vennesland, (1936); Reed, Anderson and Mendel, (1932)).

Finally, it may be noted that the results presented here appear to differ from those obtained using *in vitro* techniques. For example, Brady, Lukens and Gurin (1951) found that liver slices of hypophysectomized rats incorporated considerably more radioactive acetate into fatty acids than control groups. Conversely these investigators found that injection of growth hormone into Houssay cats (hypophysectomized-depancreatectomized animals) resulted in a profound inhibition of fatty acid synthesis by liver slices. No explanation of these differences is offered at this point.

SUMMARY

The incorporation of C^{14}-carboxyl labelled acetate into carcass and liver fatty acids was measured in growing and adult hypophysectomized, adrenalectomized, thyroidectomized and ovariectomized female rats by measuring the C^{14} content of liver and carcass fatty acids one-half hour after an intraperitoneal injection of radioactive acetate.

The hypophysectomized animals showed a marked decrease of C^{14} incorporation into liver fatty acids and a normal rate of incorporation into carcass fatty acids. This was reflected in the lower proportion of liver synthesis to total synthesis.

The adrenalectomized rats did not exhibit any significant changes in lipogenesis but the proportion of liver lipogenesis to total lipogenesis was significantly decreased.

No significant difference was found in acetate incorporation into carcass and liver fatty acids of the adult thyroidectomized animals although the total incorporation was significantly depressed. In the growing animals the synthesis in liver and carcass was depressed.

No difference was found in lipogenesis rates of carcass and liver between ovariectomized rats and controls.

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AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-fourth months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1955.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1955.

AMERICAN UROLOGICAL ASSOCIATION

"*Urology Award*—The American Urological Association offers an annual award of $1000 (first prize of $500, second prize $300 and third prize $200) for essays on the result of some clinical or laboratory research in Urology. Competition shall be limited to urologists who have been graduated not more than ten years, and to men in training to become urologists.

The first prize essay will appear on the program of the forthcoming meeting of the American Urological Association, to be held at the Statler Hotel, Boston, Massachusetts, May 28–31, 1956.

For full particulars write the Executive Secretary, William P. Didusch, 1120 North Charles Street, Baltimore, Maryland. Essays must be in his hands before December 1, 1955."

AMERICAN PSYCHOSOMATIC SOCIETY

The American Psychosomatic Society will hold its Thirteenth Annual Meeting at the Sheraton Plaza Hotel in Boston on Saturday and Sunday, March 24 and 25, 1956.

The Program Committee would like to receive titles and abstracts of papers for consideration for the program no later than December 1, 1955. The time allotted for the presentation of each paper will be twenty minutes.

Abstracts should be submitted in sextuplicate, for the Program Committee's consideration, to the Chairman, at 551 Madison Avenue, New York 22, New York.

STANLEY COBB, M.D.
Chairman, Program Committee